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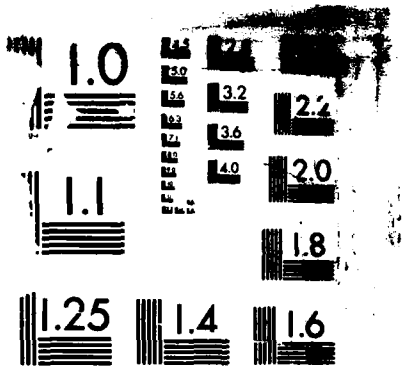
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ABSTRACTS OF PAPERS PRESENTED AT THE FORTIETH ANNUAL MEETING OF THE SOCIETY OF GENERAL PHYSIOLOGISTS

Marine Biological Laboratory

Woods Hole, Massachusetts

4–7 September 1986

INVITED PAPERS

1. The Plasma Membrane in the Regulation of Cellular Calcium ERNESTO CARAFOLI, *Swiss Federal Institute of Technology, Zurich, Switzerland*

2. The Role of the Plasma Membrane and Intracellular Organelles in Synaptosomal Calcium Regulation DAVID G. NICHOLLS, *Neurosciences Research Group, Ninewells Medical School, Dundee University, Dundee, Scotland*

Isolated nerve terminals (synaptosomes) provide a model system for studying the interaction between plasma membrane and mitochondrial calcium transport pathways. Various groups have shown that the cytosolic free Ca^{2+} is maintained below 1 μM when the plasma membrane is polarized. The plasma membrane possesses both a Ca^{2+}-translocating ATPase and an Na^+/Ca^{2+} exchange activity. The former is the dominant pathway for the extrusion of Ca^{2+} from the terminal. The latter does not contribute significantly to the extrusion of Ca^{2+} and indeed may operate in the direction of Ca^{2+} uptake in the isolated terminal. The activation of voltage-dependent Ca^{2+} channels upon depolarization increases the cytosolic free Ca^{2+} sufficiently to induce the exocytosis of GABA and L-glutamate from the terminals. With chronic depolarization, the excess Ca^{2+} is accumulated within the mitochondria. However, the release of intramitochondrial Ca^{2+} into the cytosol does not induce exocytosis. It is proposed that the role of the mitochondrion in the terminal is to act as a central sink for the radial entry of Ca^{2+}, which initiates exocytosis. The alternative role which has been proposed, that mitochondrial Ca^{2+} transport is directed toward the regulation of matrix Ca^{2+}-activated enzymes, is not supported by the finding that pyruvate dehydrogenase activity in the physiological milieu of the intact synaptosome is independent of Ca^{2+}.

3. Multiple Types of Calcium Channel in Neurons and Muscle Cells and Their Modulation by Neurotransmitters R. W. TSIEN, C. D. BENHAM, A. P. FOX, P. HESS, D. LIPSCOMBE, E. W. McCLESKEY, D. V. MADISON, and R. L. ROSENBERG, *Department of Physiology, Yale University School of Medicine, New Haven, Connecticut*

Since calcium entry through voltage-gated Ca channels is a vital link between membrane depolarization and diverse cellular responses, there has been considerable interest in the existence of multiple types of Ca channels. Patch-clamp recordings in cultured chick sensory neurons reveal three types of Ca channel with different kinetics and conductance properties that we call T, N, and L (Nowycky et al. *Nature.* 316:440). The following table gives some selected examples of the varied expression of these kinds of Ca channels in other cells:

Rat DRG	T	N	L	DuPont et al., 1986
Rat hippocampal	T	?	L	(D.V.M.); Brown et al., 1985
Rat sympathetic*	—	N	L	Hirning et al., 1986
Frog sympathetic*	—	N	L	(D.L.)
Rat type 2 astrocyte	T	—	—	Barres et al., 1985
Rat pituitary (GH3)	T	—	L	Armstrong and Matteson, 1984
Guinea pig/dog heart	T	—	L	Nilius et al., 1985; Bean, 1985
Rabbit artery smooth	T	—	L	Aaronson et al., 1986

* In sympathetic neurons, "N" labels a Ca current component that inactivates much more slowly than N-type Ca currents in chick DRG neurons but is similar in several other respects. This current plays a dominant role in mediating K-induced norepinephrine (NE) release (Hirning et al., 1986). Examples of adrenergic modulation of N- and L-type Ca channels have been found. β-Adrenergic stimulation enhances L currents in heart cells; NE acting on α-receptors increases L currents in arterial smooth muscle cells; NE decreases the inactivating current (presumably N) in frog sympathetic neurons.

4. Alterations in Intracellular Ca Produced by Changes in $[Na]_i$ and $[H]_i$ LORIN J. MULLINS, *Department of Biophysics, University of Maryland School of Medicine, Baltimore, Maryland*

Conventional theories of Na/Ca exchange require that the intracellular Na concentration be a critical factor in setting the level of ionized Ca in cells. Indeed, much experimental information supports this expectation. Our studies of Ca entry with depolarization have shown that Ca entry is not detectable if $[Na]_i$ is $\leq$10 mM and that Ca entry is saturated if $[Na]_i$ is $\geq$32 mM. Changing pH_i is known to affect Na/Ca with decreases in pH inhibiting and increases in pH enhancing Ca fluxes. Acidity also appears to raise $[Ca]_i$ by releasing stored Ca, and a change to alkaline pH lowers $[Ca]_i$, presumably by forcing Ca into stores.

5. Cellular Calcium Compartmentalization and Movements: Studies with Electron Probe X-Ray Microanalysis ANDREW P. SOMLYO and AVRIL V. SOMLYO, *Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, Pennsylvania*

The distribution of Ca^{2+} in intact cells can be determined at better than 10 nm resolution through electron probe X-ray microanalysis (EPMA) of cryo-sections (Somlyo. 1985. *Cell Calcium.* 6:197). We have used these methods to obtain information about the compartmentalization and translocations of Ca^{2+} in cells. In striated muscle, ~60% of the total Ca in the terminal cisternae (TC) is released during a tetanus, and is accompanied by the entry of K^+ and Mg^{2+} into the SR (Somlyo et al. 1985. *J. Biol. Chem.* 260:6801). The total Ca^{2+} released (~1 mM) is sufficient to saturate the Ca^{2+}-binding sites on troponin and parvalbumin. The post-tetanic return of Ca^{2+} to the TC consists of a fast and slow phase that are rate-limited, respectively, by Ca^{2+} pumping by the SR ATPase and by the off rate of Ca^{2+} from parvalbumin. In smooth muscle, transmitters release Ca^{2+} from junctional (Bond et al. 1984. *J. Physiol.* [*Lond.*]. 355:677) and central SR (Kowarski et al. 1985. *J. Physiol.* [*Lond.*]. 366:153). Ca^{2+} accumulation was also demonstrated within the SR of isolated cardiac muscle cells (Chiesi et al. 1981. *J. Cell Biol.* 91:728). Mitochondrial Ca^{2+} has been measured in skeletal, cardiac, and smooth muscle, frog retinal rods (Somlyo and Walz. 1985. *J. Physiol.* [*Lond.*]. 358:183), rat liver, and brain cortex. The major conclusion of these studies is that normal endogenous mitochondrial Ca^{2+} is relatively low (equivalent to ~0–2.5 nmol Ca^{2+}/mg mitochondrial protein), and mitochondria do *not* make a significant contribution to the physiological regulation of cytoplasmic $[Ca^{2+}]$. Mitochondria can accumulate large amounts of Ca^{2+} in situ under pathological conditions. Such pathological Ca^{2+} accumulation, observed during massive ouabain-resistant Na^+ efflux from Na-loaded cells, is reversible. The endoplasmic reticulum (ER) is the organelle that contains the highest concentration of mobilizable Ca^{2+} in liver and is the major Ca^{2+} storage site in nonmuscle

cells (e.g., retinal rods). EPMA and rapid freezing have also shed light on intracellular Ca^{2+} transport in the retina. There was no significant change in total Ca^{2+} in retinal rod outer segments during illumination, in agreement with ion-selective electrode measurements (Gold. 1986. *Proc. Natl. Acad. Sci. USA.* 83:1150). Finally, EPMA showed the presence of high concentrations of Ca^{2+} in the atrial granules storing atrial natriuretic factor (unpublished observations with R. Broderick), and demonstrated the compartmentalization of Ca^{2+} within vacuoles in sickled human red blood cells (Lew et al. 1985. *Nature [Lond.].* 315:586). [Supported by HL15835 to the Pennsylvania Muscle Institute and by Training Grant HL07499.]

6. Mechanisms Involved in Receptor-mediated Changes of Intracellular Ca^{2+} in Liver JOHN R. WILLIAMSON, CARL A. HANSEN, KATHLEEN E. COLL, and MICHAEL T. WILLIAMSON, *Department of Biochemistry and Biophysics, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania*

The effects of a variety of Ca^{2+}-mobilizing hormones on hepatic inositol lipid metabolism were investigated using [^{3}H]inositol-labeled hepatocytes. Levels of [^{3}H]inositol phosphates were measured using HPLC while changes of intracellular free Ca^{2+} were monitored with quin-2. Vasopressin (VP), phenylephrine (PE), epidermal growth factor (EGF), glucagon, and 8-Br-cAMP at maximal concentrations mobilized intracellular Ca^{2+} nonadditively from the same precursor pool. The effects of EGF, glucagon, and 8-Br-cAMP on increases of cytosolic free Ca^{2+} were smaller and slower than those of VP and PE. Each agonist increased the levels of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4, and the latter's degradation product, Ins(1,3,4)P_3, but the effects of VP were by far the greatest. Pretreatment of hepatocytes with phorbol myristate acetate (PMA) to fully activate C-kinase prevented these changes of Ca^{2+} and inositol phosphates (with VP only at a low concentration). These studies indicate that there is a poor correlation between the increase of cytosolic free Ca^{2+} and Ins(1,4,5)P_3 accumulation with different agonists, and suggest that a very small change of Ins(1,4,5)P_3 is sufficient to elicit Ca^{2+} mobilization. On the basis of results from these and other studies, it is suggested that an A-kinase–mediated phosphorylation promotes the interaction of a specific receptor-linked G-protein with phospholipase C with stimulation of inositol lipid metabolism, while C-kinase interacts negatively. Current experiments are pursuing the questions of possible regulation of inositol phosphate phosphatases and kinase by A-kinase or C-kinase and the physiological function of Ins(1,3,4,5)P_4. [Supported by NIH grants AM 15120 and AA 05662.]

7. Receptor-mediated Alterations of Intracellular Calcium in Pancreatic Cells IRENE SCHULZ, *Max-Planck-Institut für Biophysik, Frankfurt, Federal Republic of Germany*

8. A Chemical Link in Excitation-Contraction Coupling in Skeletal Muscle JULIO VERGARA, KAMLESH ASOTRA, and MICHAEL DELAY, *Department of Physiology, University of California at Los Angeles, Los Angeles, California*

We have recently obtained evidence for a biochemical coupling mechanism in the excitation-contraction (E-C) coupling process in skeletal muscle in which inositol (1,4,5) trisphosphate ($InsP_3$) is proposed to act as a soluble internal transmitter (Vergara et al. 1985. *Proc. Natl. Acad. Sci. USA.* 82:6352). New evidence includes the following. (*a*) HPLC analysis of aqueous extracts from fast-frozen stimulated and control sartorius muscles indicate that control muscles contain neglible amounts of $InsP_3$ and that inositol phosphate concentrations increase when the muscles are frozen after 5–20 s of tetanic stimulation at 50 Hz. (*b*) The polyphosphoinositides PIP and PIP_2 are readily observable in t-tubular membranes isolated from skeletal muscle. (*c*) The application of $InsP_3$ to mechanically or chemically skinned muscle fibers causes Ca^{2+} release and tension development. Concentrations of $InsP_3$ as low as 3 μM have been found to produce

contractions in saponin-treated fibers at appropriate experimental conditions. (*d*) Possible pharmacological inhibitors of $InsP_3$ formation and activity, such as the polyamine neomycin, block E-C coupling, i.e., prevent T-system depolarization from releasing Ca^{2+} in muscle fibers at physiological conditions. (*e*) The coupling process at the triadic junctions in skeletal muscle fibers is characterized by a significant latency between the depolarization of the t-tubule and the release of Ca^{2+} from the SR. This time interval, the triadic delay, is sufficiently long to allow for the participation of a chemical process. The strong temperature dependence of the triadic delay (Q_{10} near 2.7) suggests that a sequence of chemical steps may act as a link in the E-C coupling. [Supported by USPHS grant AM 25201 and by an MDA grant-in-aid.]

9. Cytoplasmic Ca^{2+} and Intracellular pH in Rat Lymphocytes SERGIO GRINSTEIN, *Department of Cell Biology, Hospital for Sick Children, Toronto, Ontario, Canada*

The relationship between the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the intracellular pH (pH_i) of thymic lymphocytes will be discussed. Elevation of $[Ca^{2+}]_i$ results in alterations of pH_i. In Na^+-containing media, treatment of the cells with ionomycin, a divalent cation ionophore, induced a moderate cytoplasmic alkalinization. In the presence of amiloride or in Na^+-free media, an acidification was observed. This acidification is at least partly due to H^+ (equivalent) uptake in response to membrane hyperpolarization since: (*a*) it was enhanced by pretreatment with conductive protonophores, (*b*) it could be mimicked by hyperpolarization with valinomycin, and (*c*) it was decreased by depolarization with K^+ or gramicidin. In addition, activation of metabolic H^+ production also contributes to the acidification. The alkalinization is due to Na^+/H^+ exchange inasmuch as it is Na^+ dependent, amiloride sensitive, and accompanied by H^+ efflux and net Na^+ gain. A shift in the pH_i dependence of transport underlies the activation of the antiport. The effect of $[Ca^{2+}]_i$ on Na^+/H^+ exchange was *not* associated with redistribution of protein kinase C and was also observed in cells previously depleted of this enzyme. Treatment with ionomycin induced significant cell shrinking, which is known to activate the antiport in these cells. Preventing shrinking largely eliminated the activation of the antiport. Therefore, stimulation of Na^+/H^+ exchange by elevation of $[Ca^{2+}]_i$ is due, at least in part, to cell shrinking and does not require stimulation of protein kinase C. The effects of changing pH_i on $[Ca^{2+}]_i$ were also measured. Several procedures that increased pH_i, such as addition of monensin or NH_3, or the osmotic activation of the Na^+/H^+ antiport, significantly elevated $[Ca^{2+}]_i$. This effect was dependent on the presence of extracellular Ca^{2+}, which suggests control of the plasma membrane Ca^{2+} channels by pH_i. Thus, the relationship between pH_i and $[Ca^{2+}]_i$ is reciprocal.

10. Characterization of Voltage-gated Calcium Channels in *Xenopus* Oocytes After Injection of RNA from Electrically Excitable Tissues TERRY P. SNUTCH,* JOHN P LEONARD,* JOEL NARGEOT,* NORMAN DAVIDSON,* and HENRY A. LESTER, *Divisions of Biology and Chemistry, California Institute of Technology, Pasadena, California*

In an effort to characterize voltage-gated calcium channels in electrically excitable tissues, we have isolated mRNA from a number of rat organs and injected it into *Xenopus* oocytes. Macroscopic currents through voltage-gated calcium channels were resolved when the endogenous oocyte calcium-dependent chloride current was blocked by replacing external calcium with barium and chloride with methanesulfonate. Under these conditions, we observed TTX-insensitive, cadmium-sensitive, voltage-dependent barium currents in oocytes injected with mRNA isolated from rat skeletal muscle, heart, or brain. As judged by the waveforms of the currents, their sensitivity to calcium channel agonists and antagonists, and their modulation by transmitters and intracellular messengers, several distinct calcium channel types were observed. For example, heart mRNA induced two distinct barium currents: (*a*) a transient component inactivated with a time constant of ~200 ms and was insensitive to dihydropyridine calcium channel antagonists, and (*b*) a slow component did not inactivate, was increased by BAY-K

Asterisks indicate authors who are not members of The Society of General Physiologists.

8644, and was blocked by nifedipine. The heart slow component was also modulated by isoproterenol, cAMP, and acetylcholine. In contrast, mRNA from 16-d brain induced a large, single component barium current that inactivated with a decay constant of ~650 ms. No effects were seen with dihydropyridine agonists or antagonists. The brain current was, however, increased (42 ± 5%) by the C-kinase activator phorbol dibutyrate. Taken together, these results show that we can distinguish among different types of calcium channels directed by exogenous mRNA in *Xenopus* oocytes. [Supported by grants GM-10991 and GM-29836 from DHHS, a grant from the AHA, Greater Los Angeles Affiliate, and by the NSERC of Canada.]

11. An Enzymatic Mechanism for Calcium-dependent Inactivation of Voltage-dependent Calcium Channels ROGER ECKERT, JOHN CHAD,* DANIEL KALMAN,* and DAVID ARMSTRONG, *Department of Biology, University of California at Los Angeles, Los Angeles, California*

Two different mechanisms of inactivation have been described for calcium currents activated by depolarization: one that depends directly on voltage and one that appears to be mediated by a specific action of calcium following its entry and accumulation during current flow. This calcium-dependent mechanism has been demonstrated in a wide variety of excitable cells by experimental conditions that prevent the accumulation of calcium ions inside the cell (Eckert and Chad. 1984. *Prog. Biophys. Mol. Biol.* 44:215). Thus, depolarizing sufficiently to reduce calcium influx, substituting barium for calcium, or buffering internal calcium with EGTA all reduce inactivation of calcium currents in those cells. Subsequently, single channel recordings of unitary barium currents have revealed at least two classes of calcium channels with different inactivation properties. One class inactivates rapidly at holding potentials more positive than −80 mV. The other class does not inactivate at depolarized holding potentials in intact cells, but stops responding to membrane depolarization altogether when the cytoplasmic side of the membrane is exposed to a minimal saline solution. This irreversible loss of activity, or "washout," also depends on calcium accumulation at the cytoplasmic surface, and can be inhibited by EGTA but not prevented. However, recent experiments on dialyzed snail neurons in our laboratory have demonstrated that washout can be eliminated completely without any calcium buffers when ATP and the catalytic subunit of cyclic AMP-dependent protein kinase are added to the dialysate in the presence of a proteolytic enzyme inhibitor. Under those conditions, calcium currents exhibit reversible calcium-dependent inactivation that can be accelerated by adding an exogenous calcium- and calmodulin-dependent phosphatase (calcineurin) purified from mammalian brain. We propose that calcium-dependent inactivation results from the activation of a calcium-dependent phosphatase, and that inactivation is removed as dephosphorylated sites are rephosphorylated by an endogenous protein kinase. Further experiments on intact molluscan neurons and cell-free membrane patches from a mammalian cell line (GH3) support that conclusion. [Supported by USPHS grant NS-8364.]

12. Regulation of Neuronal Calcium Channels and Intracellular Calcium by Protein Kinase C and Inositol Trisphosphate LEONARD K. KACZMAREK, *Departments of Pharmacology and Physiology, Yale University School of Medicine, New Haven, Connecticut*

The bag cell neurons of *Aplysia* have served as a model system for the investigation of long-lasting changes in neuronal excitability. Although these cells normally have relatively negative resting potentials and display no spontaneous activity, brief synaptic stimulation triggers a long-lasting discharge, which is associated with a significant enhancement in the height and width of action potentials. In vivo, this transformation of the properties of the bag cell neurons serves to evoke a series of reproductive behaviors. One of the biochemical events that can be detected at the onset of a discharge is the stimulation of the incorporation of [^{3}H]inositol into inositol-containing phospholipids and into inositol polyphosphates—a pathway that is believed to regulate the activity of the calcium/phospholipid-dependent protein kinase (protein kinase C) and to generate the second messenger, inositol trisphosphate. We have tested the electrophysiological effects of activation of protein kinase C by exposure of isolated bag cell neurons to

activators of protein kinase C (synthetic diacylglycerols and phorbol esters) and by direct microinjection of protein kinase C into the somata of these isolated neurons. Each of these manipulations increases the height of evoked action potentials. Voltage-clamp experiments, using internally dialyzed neurons, have demonstrated that the activation of protein kinase C enhances the voltage-dependent calcium current. Moreover, single channel recordings have shown that this enhancement of calcium current does not occur through an effect on the properties of calcium channels that can be recorded in unstimulated cells, but rather results from the unmasking of a previously covert species of calcium channel, with a unitary conductance of ~24 pS, which is never observed in control cells. In contrast, intracellular injection of the second messenger, inositol trisphosphate, does not enhance action potentials, but causes a transient hyperpolarization, which is likely to result from a transient release of calcium ions from intracellular stores. Current evidence therefore suggests that both the protein kinase C and the diacylglycerol second-messenger pathways may act to enhance intracellular calcium levels, but through different mechanisms, and that these processes act synergistically to alter the electrical properties of the bag cell neurons and to promote the release of neuroactive peptides, which occurs at the onset of a discharge.

13. Calcium in Heart Muscle Function Assessed Using Digital Imaging Fluorescence Microscopy and Fura-2 W. J. LEDERER, M. B. CANNELL, and J. R. BERLIN, *Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland*

Intracellular calcium was measured in enzymatically dissociated single rat heart cells under voltage-clamp control. Fura-2 acid was injected through the single pipette that also acted as the voltage-clamp electrode. Two excitation wavelengths (340 and 380 nm) were used to illuminate the preparation and fluorescence images at 510 nm were recorded using an ISIT video camera and a microcomputer-based image-processing system. An image of the distribution of calcium within a cell is generated by taking the ratio of intensity values of the 340-nm image to the 380-nm image after appropriate background subtraction. Complementary data were obtained at much higher temporal resolution (but with little spatial resolution) using a photomultiplier tube to measure the fluorescence intensity. The calcium transient in cells under normal conditions rises rapidly to a level of ~1 μM and declines relatively slowly (the halftime of decay is ~120 ms) to a resting level of between 50 and 100 nM. These changes of intracellular calcium occur with good spatial uniformity. The depolarization threshold for producing the calcium transient is between −50 and −45 mV, with the largest calcium transient seen at a depolarization potential of about −10 mV. Under conditions of "calcium overload," spontaneous increases of $[Ca^{2+}]_i$ can be observed. These increases can either be synchronous or propagating waves of increased $[Ca^{2+}]_i$ that traverse the cell. At normal resting potentials, these increases in $[Ca^{2+}]_i$ activate an inward membrane current that appears similar to the transient inward current (I_{Ti}) reported in other cardiac preparations.

14. Mechanism of Activation of Protein Kinase C: Role of Diacylglycerol and Calcium Second Messengers YUSUF A. HANNUN and ROBERT M. BELL, *Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina*

The mechanism of activation of the Ca^{2+}- and phospholipid-dependent protein kinase, protein kinase C, was investigated in vitro using a mixed micellar assay (Hannun et al. *J. Biol. Chem.* 260:10039). Activity was independent of the number of Triton X-100/lipid mixed micelles present. Activation was strongly dependent on the mole percent of phosphatidylserine (PS) and sn-1,2-dioleoylglycerol ($diC_{18:1}$), and on the free Ca^{2+} level. Detergents other than Triton X-100 also supported enzyme activity in the presence of Ca^{2+} and the lipid cofactors, which indicates that the detergent micelle provides an inert surface into which the lipid cofactors partition. The $diC_{18:1}$ dependence was hyperbolic and was strongly modulated by the levels of Ca^{2+} and PS present. The PS dependence, on the other hand, was highly cooperative, with a Hill number near 4. The PS requirement was also modulated by Ca^{2+} level and by the mole

percent of $diC_{18:1}$, but there was a minimum requirement of at least four molecules of PS per micelle before any activity could be detected. The affinity of the enzyme for Ca^{2+} was also modulated by the mole percent of PS and $diC_{18:1}$ present. Studies with phorbol diesters showed interactions with PS and Ca^{2+} similar to those with $diC_{18:1}$. Gel filtration studies demonstrated that monomeric protein kinase C interacts with one mixed micelle and binds phorbol diester in a ratio of 1:1. These results indicate the following minimal stoichiometry for the activation of protein kinase C: one molecule of diacylglycerol (or phorbol diester), and four molecules of PS and Ca^{2+}. A model of the interaction of Ca^{2+} with the lipid cofactors is discussed. Studies with inhibitors of protein kinase C identified sphingosine and lysosphingolipids as potent and reversible inhibitors of the enzyme in vitro and in human platelets. These molecules inhibit enzyme activity and phorbol diester binding by interacting with the regulatory subunit of protein kinase C. Sphingosine and its analogues may function as physiologic negative effectors of protein kinase C. [Supported by NIH grant AM20205 and American Cancer Society grant B5-11.]

15. Role of Ca^{2+} in the Interconversion of Pyruvate Dehydrogenase in Isolated Cardiac Myocytes RICHARD G. HANSFORD,* *National Institute on Aging, Gerontology Research Center, Baltimore, Maryland*

We have sought to assess the importance of the Ca ion in the activation of pyruvate dehydrogenase, which occurs when the workload of the heart is raised. The proportion of pyruvate dehydrogenase existing in the active form (PDH_A) in suspensions of unstimulated rat cardiac myocytes oxidizing glucose was found to be ~30%. Depolarization of the cells with concentrations of K^+ above physiological values led to an increase in the content of PDH_A: overloading of the cells with Na^+ by treatment with veratridine plus ouabain gave the same result. Each of these procedures was shown in experiments with quin-2–loaded myocytes to lead to an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$). Treatment of the cells with ruthenium red, an inhibitor of mitochondrial Ca^{2+} transport, largely prevented an increase in PDH_A in response to addition of KCl or of veratridine plus ouabain. Ruthenium red did not attenuate the increase in $[Ca^{2+}]_c$, which was shown to occur under these conditions. By contrast, treatment of the cells with ryanodine, an inhibitor of sarcoplasmic reticulum Ca^{2+} transport, or loading with the Ca^{2+}-chelating agent quin-2, did not diminish the response of PDH_A content to interventions that raise $[Ca^{2+}]_c$: the cells, however, remained totally quiescent. It is concluded that an increase in $[Ca^{2+}]_c$ causes an increase in the PDH_A content of cardiac myocytes independently of an increase in mechanical work. In the normal physiological situation, the activation of dehydrogenases by Ca^{2+} is thought to help maintain the balance of energy supply and demand during periods of increased workload, which in muscle are associated with an increased $[Ca^{2+}]_c$.

16. The Role of Microfilament Organization in Vasopressin Action DENNIS A. AUSIELLO, JOHN HARTWIG, and DENNIS BROWN, *Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts*

The stimulation of target cells in kidney collecting duct and in amphibian epithelia with vasopressin results in a marked increase in transepithelial water flow. In order for this physiological effect to be achieved, significant changes in cellular geometry and intracellular vesicle cycling must first be accomplished. This is, at least in part, achieved by alterations in microfilament organization in the subapical membrane area of the cell. This process may be facilitated by gel-sol transformation of actin filaments, a calcium-stimulated process. In order to determine the molecular components involved in the dynamic changes of cytoplasm and membrane architecture, we have begun to define the 3-D organization of microfilaments in vasopressin-sensitive cells and its relationship to vesicle cycling. Our data have revealed that toad bladder epithelia and kidney collecting duct cells contain the actin-associated regulatory proteins, actin-binding protein, gelsolin, and/or villin. Actin-binding protein is a large molecule that binds to

and cross-links actin filaments into isotropic networks that are abundant in the subapical area of both cell types. Gelsolin is a calcium-activated protein that severs and binds the high-affinity end of actin filaments. Villin has all the functions of gelsolin and in addition has the ability to bundle actin filaments in the absence of calcium. We have demonstrated that immunogold localization of actin, actin-binding protein, gelsolin, and myosin occurs in the subapical areas of renal collecting duct cells. The presence of actin and actin-regulatory proteins provides vasopressin-sensitive cells with the capacity to undergo calcium-regulated gel-sol transitions. These changes are probably involved in both the exocytotic insertion of water channels into the apical membrane, as well as in the observed alterations in cytoplasmic geometry that may be important in dictating the transcellular pathway for water movement across these cells.

17. Multiple Roles of Calcium in Anoxic-induced Injury in Renal Proximal Tubules LAZARO J. MANDEL, *Department of Physiology, Duke University Medical Center, Durham, North Carolina*

Numerous investigators have suggested that Ca plays an important role in the irreversible cell injury observed after anoxia or ischemia. Particularly, Ca seems to be involved in two phenomena that characterize irreversibly injured cells: the inability to restore mitochondrial function and plasma membrane damage. Ca accumulation in the mitochondria has been observed with irreversible cell injury, and increased cytosolic free calcium has been postulated to occur and subsequently induce a cascade of events that leads to plasma membrane damage. In the present study, we utilized a suspension of proximal tubules obtained from the rabbit kidney. The suspension was subjected to hypoxia and anoxia for 10–40 min with subsequent reoxygenation, and the effects of the perturbations on cellular Ca content, mitochondrial respiratory function, and plasma membrane integrity were evaluated. The effects of mitochondrial Ca accumulation were assessed by contrasting hypoxia with anoxia. 30 min of anoxia led to a net release of Ca from the cells, whereas 30 min of hypoxia increased Ca content three- to fourfold. This accumulation was probably mitochondrial, since it could be prevented by the addition of ruthenium red. It was also reversible within 10 min of reoxygenation. Mitochondrial respiration after 30 min of hypoxia or anoxia was equally inhibited, which shows that the Ca accumulation by itself did not cause the early cellular injury. The effects of Ca on anoxic-induced injury to the plasma membrane were assessed by lowering the extracellular Ca during anoxia. This maneuver significantly reduced the damage to the plasma membrane, as measured by release of lactate dehydrogenase, which suggests that Ca plays a role in plasma membrane damage. Preliminary measurements of cytosolic free Ca show that it does not change during short-term anoxia, which indicates that the effect on the plasma membrane may be through alterations in Ca compartmentation. [Supported by NIH grant AM-26816.]

18. Molecular Structure and Function of Calmodulin ANTHONY R. MEANS, COLIN RASMUSSEN, and JOHN A. PUTKEY, *Department of Cell Biology, Baylor College of Medicine, Houston, Texas*

Calmodulin (CaM) serves as an intracellular Ca^{++} receptor in all eukaryotic cells. A large number of Ca^{++}-dependent enzymes and cellular processes require the Ca^{++}/CaM complex as a regulatory signal. The issues to be addressed are: (*a*) What are the molecular interactions that enable CaM to regulate the wide variety of enzymes with which it interacts? (*b*) What are the physiological consequences of altering the intracellular level of CaM? Molecular interactions are examined in vitro by using bacterial expression vectors and site-directed mutagenesis to produce large quantities of the relevant Ca^{++} binding protein. These studies have led to the understanding that several CaM-dependent enzymes exhibit different molecular requirements relative to the primary amino acid sequence of CaM. Intracellular CaM levels have been altered by introducing extra copies of the CaM gene into cultured cells using an episomally carried virus vector. Constitutive or inducible increases in CaM are shown to increase the number of cells required to arrest cell growth, to decrease the duration of the cell cycle by specifically affecting

the length of G_1, and to alter the amount of both α- and β-tubulin mRNAs. Together, these approaches are being used to define the CaM target proteins that regulate cell proliferation. The overall problem of employing CaM to provide specificity to Ca^{++}-regulated processes will be addressed.

CONTRIBUTED PAPERS

19. An Intracellular Calcium Binding Site on Neurofilament Proteins of *Myxicola* Giant Axon R. F. ABERCROMBIE, K. GAMMELTOFT,* J. JACKSON,* and L. YOUNG,* *Department of Physiology, Emory University School of Medicine, Atlanta, Georgia*

It is generally accepted that some of the intracellular buffering of calcium ions in neurons is the result of calcium binding to high-affinity sites on cytoplasmic proteins. By examining cytoplasm extracted from the giant axon of the marine worm *Myxicola infundibulum*, we have determined that most of these sites are located on neurofilament (NF) proteins. The extracted cytoplasm, a gel, was liquefied by dialysis with a high-ionic-strength medium, and all particulate matter, including mitochondria and endoplasmic reticulum, was removed by centrifugation at 100,000 *g* (15 min). The soluble proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose. The nitrocellulose strips containing the proteins were incubated with $^{45}Ca^{2+}$, rinsed, and examined by autoradiography (Maruyama et al. 1984. *J. Biochem.* 95:511). This autoradiographic assay showed that a number of, but not all, intracellular proteins bound ^{45}Ca. The most prominent, by far, were the 150,000- and 160,000-mol-wt NF subunits (Gilbert et al. 1975. *Nature.* 256:596; Lasek et al. 1979. *J. Cell Biol.* 82:336). We discovered that NF proteins could be preferentially removed from the solution by freezing and thawing the sample, causing reformation of a gel, which was sedimented by centrifugation. Electrophoretic analysis showed that the 150,000- and 160,000-mol-wt proteins and a minor protein of 185,000 mol wt were preferentially removed by this procedure. Properties of cytoplasmic Ca-binding sites were examined by titrating the soluble proteins with Ca while measuring free Ca with electrodes. Titrations of solutions before and after NF protein removal showed evidence for an NF Ca-binding site with capacity 8.5 μmol/g NF protein and an affinity of 0.4 μM free Ca^{2+}. This capacity corresponds roughly to 100 μmol/kg cytoplasm. [Supported by NIH grant NS19194.]

20. Photolabile Chelators That "Cage" Calcium with Improved Speed of Release and Pre-Photolysis Affinity STEPHEN R. ADAMS,* JOSEPH P.Y. KAO,* and ROGER Y. TSIEN, *Department of Physiology-Anatomy, University of California, Berkeley, California*

Our previous chelator for "caging" Ca^{2+} was "nitr-2" (see chapter by R. Y. Tsien in *Optical Methods in Cell Physiology*, SGP symposium no. 38, P. DeWeer and B. Salzberg, editors), a nitrobenzhydrol methyl ether derived from BAPTA, a parent Ca^{2+} chelator with fast kinetics and good rejection of Mg^{2+} and H^+. Unfortunately, nitr-2 proved quite slow at releasing Ca^{2+} after a flash, with an exponential time constant of ~200 ms owing to rate-limiting loss of methanol from a hemiketal reaction intermediate. We have now succeeded in synthesizing "nitr-5," which has an −OH group where nitr-2 had an −OMe. Nitr-5 photolyzes and releases Ca^{2+} much faster, with a time constant of ~0.3 ms, with water as the unimpeachably nontoxic byproduct instead of methanol. Other properties remain similar to nitr-2, e.g., dissociation constants (K_d) for Ca^{2+} of 0.145 and 6 μM at 0.1 M ionic strength before and after photoconversion, absorption peak at 350–360 nm with an extinction coefficient of ~5,500 $M^{-1}cm^{-1}$, and quantum efficiencies of 0.01–0.1. Further strengthening of Ca^{2+} binding was desirable and was accomplished in "nitr-7," whose K_d's for Ca^{2+} are about threefold lower than those of nitr-

5, while maintaining good rejection of Mg^{2+}, with a pre-photolysis dissociation constant of ~6 mM. However, nitr-7 has a much more laborious synthesis and a slower Ca^{2+} release rate, with a time constant of ~1.8 ms. The ability to make very fast Ca^{2+} concentration jumps in the physiological range is proving highly useful in our lab and others (see, for example, Abstract 55). [Supported by NIH grants GM31004 and EY04372 and by the Searle Scholars Program.]

21. Characterization of the Electrogenic Calcium Pump in Basolateral Membrane Vesicles from Rat Parotid Gland INDU S. AMBUDKAR* and BRUCE J. BAUM,* *Clinical Investigations and Patient Care Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland* (Sponsor: William B. Guggino)

The ATP-driven Ca^{2+} pump of inverted basolateral membrane vesicles (Takuma and Baum. 1985. *Biochem. J.* 227:239) was further characterized to identify possible ion fluxes related to the Ca^{2+} transport activity. The system has a high affinity for Ca^{2+} (K_m, 60 nM) and a high transport capacity (V_{max}, 45 nmol Ca^{2+}/min/mg·protein). Mg^{2+} is an obligatory requirement for the Ca^{2+} transport activity. The initial rate of Ca^{2+} uptake is significantly altered by changing the membrane potential. The generation of a positive (intravesicular) potential inhibits Ca^{2+} transport, while a negative potential stimulates this process. Ca^{2+} transport is sensitive to both a K^+ diffusion potential and an H^+ gradient. In membrane vesicles equilibrated with 150 mM KCl, replacement of K^+ or Cl^- in the incubation medium by the impermeant ions tetramethylammonium or gluconate, respectively, decreases initial rates of Ca^{2+} transport by ~30%, as compared with the rate obtained in the presence of KCl. When both K^+ and Cl^- are replaced, an additive inhibition is observed. When KCl is substituted with equimolar KNO_3 in the incubation medium or when vesicles are equilibrated with K-gluconate before assay in the gluconate medium, control (KCl) rates of transport are obtained. Initial rates of transport are not altered by the inclusion of 5 mM phosphate or oxalate in the assay medium. Phosphate, however, does increase the steady state Ca^{2+} loading by ~30%. When vesicles are incubated with 1 mM furosemide, steady state Ca^{2+} uptake, but not the initial rate, is inhibited up to 50%. These data are consistent with the ATP-driven Ca^{2+} extrusion from parotid cell basolateral membranes being an electrogenic process, which may be electrically coupled to K^+, Cl^-, and H^+ fluxes. This system could, therefore, be modulated by any condition that alters membrane potential and/or related ion fluxes.

22. Volume- and pH-activated Ion Fluxes in *Amphiuma* Red Blood Cells JOSEPH S. ADORANTE* and PETER M. CALA, *Department of Human Physiology, School of Medicine, University of California, Davis, California*

In response to osmotic perturbation, the *Amphiuma* red blood cell exhibits volume-sensitive net ion fluxes, which tend to restore cell volume. Cell swelling activates electroneutral K/H exchange, whereas osmotic shrinkage activates electroneutral Na/H exchange (Cala. 1980. *J. Gen. Physiol.* 76:683). Exposure of *Amphiuma* red blood cells to millimolar *N*-ethylmaleimide (NEM) resulted in net K loss, which we have identified as electroneutral K/H exchange. While the volume- and NEM-induced pathways share kinetic characteristics, they differ in their response to Cl replacement by other anions. On the basis of (*a*) the difference in the anion sensitivity of K/H exchange when activated by NEM or volume and (*b*) the observation that, after activation, anion replacement is no longer inhibitory to volume-stimulated K/H exchange, we concluded that the anions are inhibitory to activation, not translocation by the K/H exchange pathway. Some, but not all, of the anion effects upon alkali metal/H exchange are secondary to anion-dependent changes in cell volume and pH. In studies where volume was carefully controlled, we were able to study anion-dependent changes in intracellular pH and its effects upon both K/H and Na/H exchange. Changes in anion composition that led to net Cl/HCO_3 exchange flux and alkaline shifts in intracellular pH resulted in graded stimulation of K/H exchange and inhibited Na/H exchange. In addition to these pH-dependent effects, NO_3 and SCN appeared to exert pH- (and volume-) independent, inhibitory effects upon the pathways

responsible for activating Na/H and K/H exchange (see also Parker. 1984. *J. Gen. Physiol.* 84:789). [Supported by HL-21179 to P.M.C. and HL/GM-06917 to J.S.A.]

23. An Endogenous Cyclic AMP-dependent Protein Kinase Modulates the Activity of Voltage-dependent Calcium Channels DAVID ARMSTRONG, *Department of Biology, University of California at Los Angeles, Los Angeles, California*

The loss of calcium channel activity from excitable membranes exposed to minimal saline solutions at their cytoplasmic surface can be prevented completely in cell-free membrane patches from a mammalian tumor cell line (GH3) by the catalytic subunit (CS) of the cyclic AMP-dependent protein kinase and the magnesium salt of adenosine triphosphate (ATP-Mg) when they are added together in the presence of calcium buffer and a protease inhibitor (Armstrong and Eckert. 1985. *J. Gen. Physiol.* 86:25*a*). Neither CS alone nor the calcium buffer (5 mM EGTA or BAPTA, $pCa^{2+} = 8$) and the protease inhibitor (0.1 mM leupeptin) by themselves prevent the loss of activity. Furthermore, the loss of activity and its restoration by protein phosphorylation are specific to the dihydropyridine-sensitive calcium channels that have a conductance near 23 pS in 90 mM Ba^{2+}, activate at voltages more positive than −20 mV, and do not inactivate at −40 mV. I now report that ATP-Mg (2 mM) alone often delays the loss of such calcium channel activity in cell-free membrane patches from GH3 cells; however, unlike the effects observed with CS plus ATP-Mg, ATP-Mg alone never completely prevents the loss of activity. In addition, its ability to delay the loss of activity varies widely between patches. Since the effect of ATP-Mg was not mimicked by a nonhydrolyzable analogue, adenylyl (β,γ,methylene)-diphosphonate (AMP-PCP), and since its effect was blocked by the specific CS inhibitor protein (PKI) (Walsh et al. 1971. *J. Biol. Chem.* 246:1977), it is likely that ATP-Mg produces its effects on calcium channel gating in cell-free patches through an endogenous cyclic AMP-dependent kinase bound to the membrane. Cyclic AMP-dependent protein phosphorylation can be stimulated in intact GH3 cells by vasoactive intestinal peptide (VIP) (Drust et al. 1982. *J. Biol. Chem.* 257:3306), and VIP (20 nM) increases calcium channel activity in cell-attached patches. The results of these experiments are all consistent with the idea that there are sites in the membrane, closely associated with each dihydropyridine-sensitive calcium channel, that must be phosphorylated in order for the channels to respond to membrane depolarization. Dephosphorylation of those sites, not voltage, would inactivate the channels and produce an irreversible loss of activity in the absence of rephosphorylation. [Supported by USPHS NS-8364 and the Los Angeles Affiliate of the American Heart Association. I am also grateful for the gifts of CS from A. Nairn, PKI from K. Diltz, and VIP from T. Bartfai, and to R. Eckert, J. Chad, and C. Erxleben for their many contributions to this work.]

24. Measurements of Ca^{++} in Single Smooth Muscle Cells Using Fura-2 and a High-Time-Resolution Microfluorimeter P. L. BECKER,* J. F. HATCH,* K. E. FOGARTY,* and F. S. FAY, *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts*

A high-time-resolution, dual-wavelength microfluorimeter has been developed to study the dynamics of intracellular calcium concentration changes in fura-2–loaded single smooth muscle cells in response to a variety of stimuli. An optical disk mounted on the shaft of a rapidly spinning motor controls the excitation wavelength of a fluorescence microscope. The disk was manufactured to allow peak transmission of 340 nm light during half of its rotation and 380 nm transmission during the other half of rotation. Fluorescence emission at 500 nm is detected during 340 and 380 nm excitation by a photomultiplier/photon-counter interfaced to a PDP 11/73 microcomputer. Cytoplasmic $[Ca^{++}]$ is calculated at each revolution of the disk by comparing the ratio of fluorescence at 340/380 to similar ratios obtained with a fura-2–free acid solution in the absence and presence of calcium. Using this device, intracellular calcium has been found to rise rapidly upon electrical stimulation from its resting value of ~90–200 nM

to ~500–1,000 nm with a $t_{1/2}$ of <500 ms. $[Ca^{++}]_i$ peaks within several seconds and then declines slowly. The rise in $[Ca^{++}]_i$ always precedes contraction of the cell. The device is presently being modified to allow simultaneous measurements of cell force, length, and membrane potential. [Supported in part by grants from NIH (HL14523 and AM07807) and the MDA.]

25. The Effect of *N*-Ethylmaleimide (NEM) on K Transport in Human Erythrocytes of Different Ages LEE R. BERKOWITZ* and EUGENE P. ORRINGER,* *University of North Carolina at Chapel Hill, Chapel Hill, North Carolina* (Sponsor: John C. Parker)

The ability of NEM to induce chloride-dependent K transport in high-K sheep red cells is dependent on cell age, as only young cells, not old cells, exhibit loss of KCl after NEM exposure. We investigated whether such an age-dependent response to NEM was present in human erythrocytes. To obtain discrete populations of young and old cells, the density centrifugation method of Murphy (1973. *J. Lab. Clin. Med.* 82:334) was used, in which young cells comprise the top 5% of the post-centrifugation cell population and old cells comprise the bottom 5% of the centrifuged cells. We found that when these separated red cells were exposed to 0.5 mM NEM, young cells lost 50% of cell K, compared with 10% K loss in old cells. In young and old cells, NEM-induced K loss was not seen when intracellular chloride was replaced by nitrate. For both subpopulations, 1 mM furosemide inhibited 75% of the NEM-induced K flux. Investigation of the dose dependence showed a half-maximal stimulation of K flux at 0.2 mM NEM in both young and old cells. Measurements of K fluxes in cells exposed to 0.5 mM NEM for variable periods of time indicated that young cells had an increase in K flux after a 1-min exposure to NEM and a half-maximal response after 3 min of NEM exposure. For older cells, up to 5 min of incubation with NEM produced no increase in K flux. A half-maximal response occurred after 10 min of NEM exposure. These data support the idea that human red cells are similar to high-K sheep red cells in their response to NEM and suggest that the cellular sulfhydryl group response for NEM-induced K loss in human red cells undergoes changes with cell aging.

26. Electron Probe Analysis of Ca Release from the Endoplasmic Reticulum, and Mitochondrial Ca Content During Vasopressin Stimulation of Rat Liver In Situ MEREDITH BOND,* GIZELLA VADASZ,* AVRIL V. SOMLYO, and ANDREW P. SOMLYO, *Pennsylvania Muscle Institute, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania*

The relative roles of endoplasmic reticulum (ER) and mitochondria as intracellular stores of hormone-releasable Ca were assessed by electron probe analysis of rat liver rapidly frozen in situ (A. P. Somlyo et al. 1985. *Nature [Lond.]*. 314:622). VP injection (100 mU, four animals, or 260 mU, one animal) of 14–25 s duration into the superior mesenteric vein caused a significant reduction in the Ca content of stacks of rough endoplasmic reticulum (RER). The Ca content was 4.2 ± 0.24 SEM nmol Ca/kg dry weight (three rats, n = 36) in non-injected controls, 3.3 ± 0.17 in sham-injected livers (three rats, n = 48), and 2.7 ± 0.18 (five rats, n = 49) in VP-injected livers. Approximately 50% of the microvolume probed within the RER stacks consists of cytoplasm; therefore, our uncorrected measurements underestimate both the total Ca in the ER, and the amount released, by about twofold. The difference in Ca content of the ER between non-injected and sham-injected (0.9 ± 0.30 SD), as well as the difference between sham-injected and VP-injected animals (0.6 ± 0.25 SD), was significant ($P < 0.05$). Thus, at least 36% of the Ca could be released from the RER. Release of Ca by sham injection alone may be due to adrenergic effects on the liver caused by stimulation of hepatic nerves. Mitochondrial Ca in non-injected controls was 1.4 ± 0.19 SEM (n = 35) and did not change significantly (analysis of variance) upon either sham (1.6 ± 0.17; n = 47) or VP injection (0.9 ± 0.19, n = 41). Both sham and VP injection caused a significant reduction (1.2 ± 0.46 SD) in total cell Ca, from 4.1 ± 0.37 (n = 14) to 2.9 ± 0.48 (n = 15) and 2.9 ± 0.30 (n = 16). These results indicate that VP releases Ca from the ER of the liver. We find no evidence of VP increasing total mitochondrial Ca. [Supported by NIH grants AM36064 to M.B. and HL15835 to the Pennsylvania Muscle Institute.]

27. Mitochondrial Ca^{2+} Uptake During Massive Cellular Na^+ Efflux and Its Reversibility In Situ: an Electron Probe Study R. BRODERICK,* A. J. WASSERMAN,* T. FUJIMORI,* and A. P. SOMLYO, *Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania*

The rapid efflux of cellular Na^+ can cause a large increase in cytoplasmic Ca^{2+} (Glitsch et al. 1970. *J. Physiol.* [*Lond.*]. 209:25; Pritchard and Ashley. 1986. *FEBS Lett.* 195:23) that may lead to pathological Ca^{2+} loading of mitochondria (Ruano-Arroyo et al. 1984. *J. Mol. Cell. Cardiol.* 16:783). In this study, the extent and reversibility of mitochondrial Ca^{2+} uptake, secondary to cellular Ca^{2+} influx stimulated by massive Na^+ efflux, was evaluated with electron probe microanalysis (EPMA). Strips of rabbit portal vein (RPAMV), mounted on an isometric tension transducer, were Na^+-loaded for 3 h at 37°C in a K^+-free, 10^{-3} M ouabain-containing solution. To induce rapid Na^+ efflux, the strips were then washed with an Na^+-free, K^+, Li^+ solution (10^{-3} M ouabain) for 10 min and flash frozen. Mitochondrial and cytoplasmic composition was determined in cryosections by EPMA. Li^+ washing of Na^+-loaded RPAMV produced a maximal contraction ($t_{1/2}$ = 8 min), and by 10 min, >90% of the accumulated (six times normal levels) cytoplasmic and mitochondrial Na^+ was lost. Mitochondrial Ca^{2+} increased >100-fold from the Na^+-loaded (normal) values (2 mmol/kg dry wt) to 329 mmol/kg dry wt (range, 0.6–1,491 mmol/kg dry wt), while phosphorus increased from 512 to 831 mmol/kg dry wt (range, 523–1,553 mmol/kg dry wt), and Mg^{2+} increased significantly to 93 mmol/kg dry wt (range, 49–212 mmol/kg dry wt). Mitochondrial Ca^{2+} accumulation was eliminated when the Li^+ wash contained either 0 Ca^{2+} (2 mM EGTA) or 0.05 mM Ca^{2+}. At 0.2 mM $[Ca^{2+}]_o$, the increase in mitochondrial Ca^{2+} was significant (to 23 mmol/kg dry wt), but much smaller than in normal (1.2 mM) $[Ca^{2+}]_o$. Mitochondrial and cellular Na^+ loss at 0, 0.05, and 0.2 mM $[Ca^{2+}]_o$ was 44, 80, and 90%, respectively. Mitochondrial Ca^{2+} sequestration was also prevented by performing the Li^+ wash at 2°C, at which temperature mitochondrial and cellular Na^+ loss was 50% within 10 min. During Li^+ washout without ouabain, the amount of Ca^{2+} accumulated by the mitochondria was reduced by 70% to 102 mmol/kg dry wt, but the extent of cellular and mitochondrial Na^+ loss was identical to that found in the presence of ouabain. To determine the reversibility of pathological Ca^{2+} loading, the effect of prolonged incubation (in the 1.2 mM Ca^{2+}, Li^+ wash solution) was determined. Mitochondrial Ca^{2+} decreased from 329 to 129 mmol/kg dry wt at 30 min, 75 mmol/kg dry wt at 60 min, and was within control levels (2 mmol/kg dry wt) by 120 min. The maximal contractile response of these tissues to K^+ plus norepinephrine was also normal. These findings directly demonstrate Ca^{2+} unloading from pathologically Ca^{2+}-loaded mitochondria in situ. [Supported by grant HL15835 to the Pennsylvania Muscle Institute, training grant HL07499, and by the Southeastern Pennsylvania Chapter of the American Heart Association.]

28. Effects of Calcium and Pentobarbital on Synaptic Transmission at the Crustacean Neuromuscular Junction D. BROSIUS,* D. MEISS, and L. M. KENNEDY, *Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York, and Biology Department, Clark University, Worcester, Massachusetts* (Sponsor: D. C. Spray)

Previous studies have shown that neuromuscular synapses on distal and proximal fibers of the distal accessory flexor muscle (DAFM) of the lobster, *Homarus americanus*, display different physiological characteristics (Meiss and Govind. 1980. *J. Neurobiol.* 11:381). Distal fibers resemble phasic crustacean muscle fibers, while proximal fibers are tonic in nature. We have further examined these differences and have studied the influence of extracellular calcium ($[Ca]_o$) and the barbiturate pentobarbital (PNTB) on synaptic transmission in the DAFM. Focal extracellular recordings show that the mean evoked excitatory synaptic current (EPSC) is larger at synapses on distal fibers than at synapses on proximal fibers. Distal synapses release more transmitter per nerve impulse than do proximal synapses. The slope of the log (EPSC) to log $[Ca]_o$ relation does not differ between distal and proximal synapses, which indicates that differences in transmitter release between synapses on these fibers may be related to a mechanism subsequent to the calcium-dependent stage in the release process. PNTB, in concentrations ranging from 80 to 2,560 μM, significantly affects transmitter release at distal synapses. Above 640 μM, PNTB reduces synaptic current at all active sites. Synaptic current attenuation may

reflect some PNTB-induced postsynaptic membrane desensitization in addition to presynaptic PNTB effects. At some distal synaptic sites, lower concentrations of PNTB potentiate synaptic current. Potentiation of the EPSC almost certainly reflects a presynaptic effect of PNTB, either on calcium influx or sequestration. We conclude that a significant aspect of PNTB's action on the crustacean neuromuscular synapse is its modulation of calcium fluxes. It is not unlikely that similar mechanisms are involved in PNTB's action on the vertebrate central nervous system.

29. Cell Volume, K Transport, and Cell Density in Human Erythrocytes CARLO BRUGNARA* and DANIEL C. TOSTESON, *Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts*

Maturation of reticulocytes and aging of red cells are characterized by a reduction in cell volume, which takes place through a loss of cell K and water. In this study, we have investigated the K transport properties and the regulation of cell volume of the least dense (reticulocyte-rich) and densest fractions of human red cells. Red cells were fractionated according to density by using discontinuous Stractan II gradients. When cell volume was increased by increasing K content (at constant cell Na, nystatin technique), erythrocytes of the least dense but not of the densest fraction shrank back toward their original volume. This process was due to a ouabain- and bumetanide- (OB) resistant K loss. OB-resistant K efflux from the least dense fraction was stimulated by hypotonic swelling and had a bell-shaped dependence on pH (pH optimum, 6.75–7.00, inhibition at pH 6.0 and 8.0). These pH and volume effects were not evident in the densest fraction. The swelling-induced K efflux from the least dense fraction was inhibited when chloride was substituted by nitrate, thiocyanate, and acetate, whereas it was stimulated by bromide. The swelling-induced K efflux from the least dense fraction was not affected when the free internal Ca was reduced by using A23187 and EGTA, or when the external Mg was increased from 0 to 10 mM. However, internal Mg markedly inhibited K efflux from isosmotically swollen cells. *N*-ethylmaleimide (NEM; 1 mM) treatment greatly increased OB-resistant K efflux from the least dense fraction but not from the densest fraction. This NEM-stimulated K efflux was not inhibited by alkaline pH or hypertonicity. These data reveal that OB-resistant K movement in the least dense fraction of human red cells is dependent on volume, pH, and chloride and is inhibited by internal Mg. It is likely that this pathway plays a role in determining the water and cation content of normal human red cells. [Supported by NIH grant HL 36076.]

30. A Volume-dependent KCl Transporter Is Highly Expressed in Young Human Red Cells with AA and SS Hemoglobin: Physiological and Pathophysiological Implications MITZY CANESSA, MARY E. FABRY,* ANDA SPALVINS,* NADIA BLUMENFELD,* and RONALD L. NAGEL,* *Endocrine-Hypertension Unit, Brigham and Women's Hospital, Boston, Massachusetts, and Division of Hematology, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York*

We report here that a KCl transporter, which is stimulated by *N*-ethylmaleimide (NEM) and cell volume increase (hypo-osmotic), exists in young red cells of individuals homozygous for hemoglobin (Hb) A (AA) and S (SS), which were separated by centrifugation in Percoll-Stractan continuous density gradients. We found a very high activity (mmol/liter cell·h = FU) of the NEM-stimulated KCl transporter in reticulocytes and young red cells with Hb SS (43 ± 27 FU, $x \pm$ SD, $n = 9$) and AA individuals (41.7 ± 10 FU, $n = 3$). The activity of the KCl transporter but not that of Na-K-Cl cotransport was found to be age dependent in AA and SS cells. Cell swelling (230 mosmol) markedly stimulated Cl-dependent K efflux (ouabain and bumetanide resistant) in SS whole blood (9.8 ± 7.4 FU, $n = 9$), in AA young cells (21.4 ± 11 FU, $n = 3$), and in SS young cells (13 ± 13 FU, $n = 7$). Using isopycnic gradients, we found increased MCHC in young SS cells that had been swollen in the presence of chloride, which activates the KCl transporter and results in a volume-regulatory decrease response (VRD). The presence of VRD in AA young cells raises the possibility that the KCl transporter is involved in the process

of volume reduction, which occurs during maturation of erythropoietic precursors. Its high activity in a large fraction of young SS cells also has pathophysiological consequences because it corrects the decrease in the intracellular concentration of Hb secondary to low pH or osmolarity, an unwelcome pro-sickling event. The large interindividual variations in the expression of the KCl transporter also suggest that it may have a role as a genetic modulator of the phenotypic expression of sickle cell anemia.

31. Calcium Fluxes Activated During Neurite Spiking Induced by Bradykinin

JOHN E. CHAD* and JANE C. YEATS,* *Sandoz Institute for Medical Research, Gower Place, London, England*

The naturally occurring nonapeptide bradykinin (BK) produces a depolarization accompanied by neurite spiking in cultured neonatal rat dorsal root ganglion (DRG) neurons, recorded using whole-cell patch techniques in the current-clamp mode. BK responses require the outgrowth of neurites from the cell bodies after plating from a dissociated cell suspension. The site of action of BK is too remote from the cell body to allow accurate space clamp. However, the action of BK to produce activity in voltage-dependent channels on neurites permits investigations of the properties of these channels. The influx of Ca into DRG neurons can be measured using the accumulation of the radioactive tracer ^{45}Ca. These experiments show that the BK-dependent entry of ^{45}Ca (25 μM) is facilitated by the presence of millimolar concentrations of external cold Ca, as would be predicted from the two-site model of Ca permeation (Hess and Tsien. 1984. *Nature [Lond.]*. 309:453). Furthermore, the Ca influx is modified by the dihydropyridines in that the Ca channel agonist Bay K 8644 enhances the influx and the antagonist nifedipine decreases the influx. Contributions to Ca influx from the propagation of activity into the cell body cannot be discounted and further investigation will address this. Thus, the predominant Ca flux, during BK-induced depolarization, through voltage-sensitive Ca channels on the neurites of DRG neurons, appears to be through channels in which the properties investigated thus far are similar to those of the L-type channels on chick DRG cell bodies (Nowycky et al. 1985. *Nature [Lond.]*. 316:440).

32. Measurement of Intracellular Free Calcium in Intact Epithelia Using Fura-2

HERBERT CHASE, JR. and SHIRLEY WONG,* *Columbia University, New York*

In sodium-transporting epithelia such as the toad bladder, intracellular free calcium ($[Ca]_i$) is thought to regulate the passive cation permeabilities of the apical and basolateral membranes. The demonstration that Ca^{++} plays a role in the regulation of ion transport requires the simultaneous measurement of $[Ca]_i$ and ion transport in an intact epithelium. To measure $[Ca]_i$, we mounted a toad hemibladder mucosal side up in a modified Ussing chamber and loaded cells by adding fura-2 ester, dissolved in Ringer's, to the mucosal side. Fluorescence was measured through a 40× water immersion lens attached to an upright scope equipped with a mercury lamp. The sample was excited at 360 and 380 nm, in succession, and the emitted light passed through a 430-nm cut-off filter. We calculated $[Ca]_i$ by measuring the ratio of the emitted light intensity of the sample when excited at 360 and 380 nm in Ringer's as well as after adding ionomycin and excess EGTA to obtain a maximum (high [Ca]) and minimum (low [Ca]) signal. Because of the low esterase activity in granular cells, we had to amplify the fluorescent signal using an SIT camera attached to a video recorder. Light intensity, measured off a monitor with a video analyzer, was a linear function of [fura-2]. To measure transepithelial sodium transport, we measured the short-circuit current (I_{sc}) with a conventional voltage clamp. Neither loading toad bladder cells with dye nor exciting the tissue with ultraviolet light had any effect on either the transepithelial potential difference or I_{sc}. In three experiments on toad bladder, $[Ca]_i = 114 \pm 29$ nM (using a K_D for the dye of 200 nM). In similar experiments using MDCK cells grown on plastic dishes (in which we did not measure transport), $[Ca]_i = 75 \pm 18$ nM ($n = 4$). The addition of 8-bromo-cyclic AMP (0.5 mM) increased $[Ca]_i$ approximately threefold (293 ± 27%, $n = 3$). These experiments demonstrate that the measurement of $[Ca]_i$ in intact epithelia is feasible and should elucidate the role of calcium in the regulation of ion transport. [Supported by NIH grant AM01090.]

33. Hypothalamic Factor Reversibly Inhibits Active Sodium Transport in Isolated Frog Skin EDWARD CHEN,* GARNER T. HAUPERT, JR.,* and HORACIO F. CANTIELLO,* *Renal Unit, Massachusetts General Hospital, Boston, Massachusetts* (Sponsor: D. A. Ausiello)

Bovine hypothalamus contains a low-molecular-weight, nonpeptidic molecule that has some of the characteristics and biological properties of the cardiac glycosides (Haupert and Sancho. 1979. *Proc. Natl. Acad. Sci. USA.* 76:4658). We studied the effect of the hypothalamic factor (HF) on the transepithelial electrical parameters in isolated skin from *Rana pipiens* in an Ussing chamber. Short-circuit current (I_{scc}, $\mu A/cm^2$), open transepithelial voltage (V_o, mV), and transepithelial resistance after a voltage clamp of ±20 mV (R_T, Ω/cm^2) were measured in frog skins bathed in a high-NaCl (115 mM) Ringer solution at pH 8.5. HF (≅4 nM) induced a rapid (apparent macroscopic $t_{1/2}(on) < 17$ min) reduction of I_{scc} from 30.1 ± 3.13 (9) to 10.5 ± 1.78 (9), which was correlated with a decrease in V_o. Like ouabain, HF inhibited only from the serosal surface and the dose-dependent decrease in I_{scc} was accompanied by an increase in R_T. In contrast to ouabain, the reversal of I_{scc} inhibition was relatively rapid after washout of HF, bringing I_{scc} to 29.0 ± 2.08 (9) with an apparent macroscopic $t_{1/2}(off) \approx 10.8$ min. A curve-fitting of the fractional ouabain-sensitive I_{scc} vs. HF concentration showed a pseudo–first-order reaction approaching one binding site for HF for each Na^+ pump with an apparent $K_d = 0.37$ nM. Maneuvers to increase the intracellular sodium concentration (vasopressin and/or nystatin) showed the HF to be in apparent equilibrium with the basolateral pumps at each concentration. We conclude that HF is a potent inhibitor of the active transport parameters in isolated frog skin, with the rapid dissociation reaction being in striking contrast to ouabain. This model provides a useful tool for studying the regulatory aspects of transepithelial transport mediated by the Na^+/K^+ pump, particularly with regard to an endogenous inhibitor. [Supported by NIH grant HL33536 to G.T.H.]

34. Acetylcholine Increases the Voltage-activated Ca^{++} Current in Freshly Dissociated Smooth Muscle Cells LUCIE H. CLAPP,* MICHEL B. VIVAUDOU,* JOSHUA J. SINGER, and JOHN V. WALSH, JR., *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts*

The voltage-activated Ca^{++} current (I_{Ca}) and its regulation by acetylcholine (ACh) were studied in single smooth muscle cells freshly dissociated from the stomach of the toad *Bufo marinus* using whole-cell recording with patch pipettes. Experiments were carried out in a bathing solution containing (in mM): 117 NaCl, 3 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 $NaHCO_3$, 11 glucose, and 5 HEPES-NaOH (pH 7.8). Patch electrodes (2–10 MΩ) were filled either with 130 CsCl, 10 EGTA, and 10 HEPES-CsOH (pH 7.2), or with 130 KCl, 5 EGTA, and 10 HEPES-KOH. Under these conditions, I_{Ca} could be recorded for up to 1 h, although its magnitude gradually decreased with time. With K^+ in the pipette, a jump of the membrane potential from a holding level of −80 mV to more positive potentials initiated an inward Ca^{++} current followed by an outward current that declined from a peak to a steady state level. With Cs^+ in the pipette, the peak in the outward current trace disappeared shortly after rupturing the patch; at command potentials of <10 mV, the inward current peaked at ~20 ms and then declined over a period of 3 s to the level of the leak. Maximum currents, which were as large as 200 pA in 1.8 mM Ca^{++} and 800 pA in 20 mM Ca^{++}, occurred near 5 mV. Substantial inactivation of I_{Ca} occurred at holding potentials negative to −35 mV, the threshold for activation, which indicates voltage-dependent inactivation. A brief application of ACh (50 or 100 μM) by pressure ejection from a micropipette caused a significant (as much as 50%) and reversible increase in the peak inward current with Cs^+ in the patch pipette and either 1.8 or 20 mM Ca^{++} in the bath. The ACh-induced increase lasted for at least 1 min after application had ceased and was also observed in the absence of external Na^+ (54 mM Ca^{++} and 45 mM TEA). These results demonstrate that, in addition to suppressing a K^+ current (M-current) in these cells (Sims et al. 1985. *J. Physiol. [Lond.].* 367:503), ACh also increases a Ca^{++} current. [Supported by NIH grant AM31620 and NSF grant DCB85411674.]

35. Developmental Changes in the Steady State Kinetic Parameters of the Calcium Current in Isolated Rat Heart Cells NERI M. COHEN* and W. J. LEDERER, *Department of Physiology, University of Maryland at Baltimore, School of Medicine, Baltimore, Maryland*

Calcium channel current (I_{Ca}) may play an important role in determining the shape and duration of the cardiac action potential. Steady state kinetic parameters (d_∞ and f_∞) of I_{Ca} have been measured in neonatal (2–7 d) and adult (6–8 wk) rat heart cells to determine whether the changes in the shape and duration of action potentials seen during development may depend on changes in I_{Ca}. Heart cells were enzymatically dissociated and voltage-clamped using the whole-cell voltage-clamp technique (Hamill et al. 1981. *Pflügers Archiv.* 391:85). The superfusion solution contained (in mM): 1 $CaCl_2$, 145 NaCl, 4 KCl, 1 $MgCl_2$, 10 glucose, 10 HEPES (pH = 7.4), and 10 μM tetrodotoxin, T = 22–23°C. The holding potential (−50 mV) was chosen to inactivate a large fraction of sodium channels and to optimize recording from only one type of calcium channel ("L-type"; cf. Nilius et al. 1985. *Nature [Lond.]*. 316:443). The pipette solution contained 129 mM K-glutamate, 20 mM CsCl, 1 mM NaCl, and 10 μM EGTA. We have found in the neonatal cells that over a range of potentials (−40 to +40 mV), both d_∞ and f_∞ are nonzero and that this overlap gives rise to a measurable steady state inward calcium current. In adult heart cells, there is an overlap of d_∞ and f_∞, but it is significantly reduced because of a shift of f_∞ to more negative potentials. We conclude that the measured changes in the steady state kinetic parameters of I_{Ca} with development could be responsible for some of the observed differences in neonatal and adult rat action potentials. [Supported by a PMA grant to N.M.C. and NIH grant HL 25675 to W.J.L.]

36. Hydrogen Transients in Fast-Contracting Mammalian Muscle CAROL COLLINS* and J. GROSSIE,* *Department of Physiology, Ohio State University, Columbus, Ohio* (Sponsor: B. A. Biagi)

Intracellular hydrogen (a_H^i) was measured at 30°C in fast-contracting muscle (rat extensor digitorum longus) with liquid H^+-sensitive electrodes, which had an average slope of 59 mV/10-fold change in a_H. At 30°C (5% CO_2, 24 mM HCO_3), the average internal pH was 7.2 ± 0.008 (n = 198 cells). After increasing a_H^i by exposure of the muscle to 20 mM Na acetate (5% CO_2, 24 mM HCO_3) or H_2CO_3 (30% CO_2, 24 mM HCO_3), reductions of a_H^i were recorded for 10–20 min upon removal of external acid. The resulting change in a_H^i was assumed to be exponential and an approximate rate constant was calculated for the mean a_H^i recorded for each group over the initial 60-s period of acid removal. Rate constants seen upon removal of acetic acid (H acet) and H_2CO_3 were similar (0.0028 s^{-1}, n = 13, and 0.0047 s^{-1}, n = 21, respectively). The substitution of 10 mM HEPES and Na gluconate for $NaHCO_3$ caused a depolarization and a decrease in a_H^i, but successive pulses of H acet caused a gradual rise in baseline a_H^i. However, the rate constant seen upon removal of H acet (0.0028 s^{-1}, n = 12) was not altered as compared with controls with normal $NaHCO_3$. The removal of Na (tetramethylammonium) from normal and H_2CO_3 Ringer did not greatly reduce the rate constant for acid recovery (0.0047 and 0.0045 s^{-1}, respectively). In normal Ringer, amiloride (2 mM), SITS (1 mM), and DIDS (0.1 mM) also failed to decrease the rate constant seen upon the removal of H_2CO_3 and return to normal Ringer. These findings suggest that Na-H, Na-HCO_3, and Cl-HCO_3 exchanges are not major modes of reducing a_H^i in this muscle. At 20°C, the rate constant seen upon the removal of H_2CO_3 was 27% higher than that observed at 30°C (n = 8), which suggests a considerable endogenous H^+ production in this muscle. [Sponsored by OSU Graduate School.]

37. Measurements of Cytoplasmic Free Calcium in *Xenopus laevis* Oocytes During Development, Maturation, and Activation R. J. CORK* and K. R. ROBINSON, *Department of Biological Sciences, Purdue University, West Lafayette, Indiana*

We are using aequorin, calcium-selective electrodes, or the fluorescent indicator fura-2 to measure free calcium ($[Ca^{2+}]_i$) in oocytes of *Xenopus laevis*. Stage VI oocytes undergo progesterone-induced maturation in vitro, but using aequorin or calcium electrodes, we have been unable to detect any changes in $[Ca^{2+}]_i$ during this phase. Activation of mature oocytes gives rise to a wave of $[Ca^{2+}]_i$ spreading over the cell (Busa and Nuccitelli. 1985. *J. Cell Biol.* 100:1325). This is easily detected using our methods. The value of $[Ca^{2+}]_i$ rises from a resting level of ~0.1 μM (92 ± 30 nM from aequorin measurements; 140 ± 50 nM from electrodes) to a peak at ~1 μM. These results are interpreted as showing that calcium is not the second messenger for hormone-induced maturation. We are presently using fura-2 to determine whether a calcium gradient exists between the animal and vegetal poles of the oocyte, and whether such a gradient plays any role in determining the polarity of the oocyte. [Supported by NSF grant PCM 8315719.]

38. Dichlorobenzamil, an Na-Ca Exchange Blocker, Slows Recovery Following Potassium Contractures BRIAN A. CURTIS, *University of Illinois College of Medicine at Peoria, Peoria, Illinois*

Twitch muscle fibers contain two contraction-initiating messenger systems, one linking transverse tubule (t) to sarcoplasmic reticulum (SR) and a second (certainly Ca) linking SR to contractile proteins. The spontaneous relaxation of a contracture probably represents the cessation of first messenger transmission. The recovery of contractile ability following repolarization represents a resetting of this first messenger system, which proceeds as a first-order reaction with a Q_{10} of 2.08; this is suggestive of an exchange or gating reaction. The recovery reaction slows in low external Ca and low pH. Dichlorobenzamil (DCB; 0.1 μM), an Na-Ca exchange blocker, slowed the rate constant of recovery from 71 s^{-1} (14) to 251 s^{-1} (13) at 15°C in bundles of three to five fibers from tibialis anterior of *Rana pipiens*. Low pH further reduced recovery. At 60 s, Ringer, pH 7.0, there was 67% recovery (19); Ringer, pH 6.3, 64% (9); DCB, pH 7.0, 37% (7); and DCB, pH 6.3, 16% (7). DCB reduced the rate of rise of contracture tension by ~10% but maximum tension and duration were unchanged. The effects of DCB were only slowly (1 h) reversible. The bundles would twitch for several hours, with twitch tension augmented and prolonged. 0.01 μM DCB gave barely perceptible changes, whereas 1 μM led to a loss of twitch and ultimately contracture tension. The slower rate of rise of tension without a reduction of either maximum tension or contracture duration is consistent with a reduced rate of presentation of a constant amount of first messenger. This effect of an Na-Ca exchange blocker suggests the first messenger might also be Ca, stored in the t-wall and liberated on the sarcoplasmic surface of the t-system by an Na-Ca exchanger, which makes only one cycle upon depolarization. The slower rate of repriming in DCB suggests that the recovery phase following repolarization is the recycling of the Na-Ca exchanger, so that the Ca site returns to a t-lumen orientation and then refills with Ca—the observed delayed Ca influx.

39. Subcellular Compartmentalization of Fura-2 in Mammalian Vascular Smooth Muscle? TIA T. DEFEO* and KATHLEEN G. MORGAN, *Department of Medicine, Harvard Medical School/Beth Israel Hospital, Boston, Massachusetts*

Using an enzymatically isolated cell preparation of the ferret portal vein loaded with the fluorescent Ca^{2+} indicator fura-2, the ratio of fluorescent intensity at 350 nm to that at 390 nm was measured within a 2-μm-diam circular area of the image of a single cell with a photomultiplier tube. Increases in the ratio could be detected either in response to 66 mM K^+ or to 10^{-7} M ionomycin, both of which caused the cells to contract. In contrast, a statistically significant decrease in the ratio was observed in response to 10 mM caffeine, an agent that is known to release Ca^{2+} from the sarcoplasmic reticulum in other smooth muscle preparations. In intact strips of ferret portal vein loaded with the luminescent Ca^{2+} indicator aequorin, 10 mM caffeine caused a reproducible increase in luminescence, which suggests that caffeine increases cytoplasmic Ca^{2+}. In an attempt to resolve the apparent discrepancy between the caffeine data obtained with fura-2 and aequorin, we examined the fura-2 video image pairs in detail. The

control fluorescent images at both 350 and 390 nm showed marked heterogeneity in intensity, but punctate bright spots were much more evident in the 350-nm image. The addition of caffeine caused a decrease in the granularity of the 350-nm image and an increase in the granularity of the 390-nm image. These data are consistent with the concept that fura-2 fluorescence originates, in part, from a caffeine-depletable Ca storage site in mammalian vascular smooth muscle cells. [Supported by NIH grant HL31704 and by an Established Investigatorship and a grant-in-aid from the American Heart Association.]

40. Binding of a Tritiated Amiloride Analogue to the Na^+/H^+ Exchanger of Rat Lymphocytes S. JEFFREY DIXON,* SARA COHEN,* EDWARD J. CRAGOE, JR.,* and SERGIO GRINSTEIN, *Department of Cell Biology, The Hospital for Sick Children, Toronto, Ontario, Canada, and Merck, Sharp & Dohme Research Laboratories, West Point, Pennsylvania*

Rat thymic lymphocytes (thymocytes) possess an active amiloride-sensitive (apparent $K_i \simeq 5$ μM) Na^+/H^+ exchanger in their plasma membrane. We used fluorescence techniques to monitor changes in cytoplasmic pH induced by hypertonic activation of the Na^+/H^+ antiport. 5-(*N*-methyl-5-*N*-isobutyl)-amiloride (MIA) was found to be a potent inhibitor (apparent $K_i \simeq 140$ nM) of Na^+/H^+ exchange. Inhibition was rapid (<5 s) and reversible. [^{3}H]MIA binding to whole cells was assayed by rapid centrifugation after short (5 s) incubations to minimize nonspecific binding. Specific binding was defined as that displaced by excess unlabeled amiloride. When 25 nM [^{3}H]MIA was used, the ratio of nonspecific to specific binding was 4:1. Scatchard analysis revealed that [^{3}H]MIA bound with high affinity ($K_d \simeq 170$ nM) to a maximum of 8,000 sites per thymocyte. In contrast, there was no significant specific [^{3}H]MIA binding to human erythrocytes, which have relatively little Na^+/H^+ exchange activity. In thymocytes, the ability of several amiloride analogues to displace [^{3}H]MIA correlated with their potency as inhibitors of the antiport. Both inhibition and binding assays revealed that, unlike amiloride, MIA was not competitive with extracellular Na^+. Specific [^{3}H]MIA binding was, however, strongly dependent on extracellular pH, decreasing twofold as the pH was lowered from 7.5 to 6. Taken together, these data suggest that [^{3}H]MIA binds to the thymocyte Na^+/H^+ exchanger. Assuming an upper limit of 8,000 functional exchangers per thymocyte, we estimate that the turnover rate of each maximally activated exchanger is at least 2,000 cycles·s^{-1}. [Supported by the Medical Research Council of Canada.]

41. Ca^{++}/Calmodulin-dependent Phosphorylation and Regulation of Rabbit Ileal Na and Cl Absorption M. DONOWITZ, M. COHEN,* M. EL-SABBAN,* E. EMMER,* J. McCULLEN,* H. MURER, and G. W. G. SHARP, *Departments of Medicine and Physiology, Tufts University School of Medicine and New England Medical Center, Boston, Massachusetts, and Department of Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, New York*

In rabbit ileum, Ca^{++}/calmodulin (CaM) appears to be involved in the regulation of the basal-linked neutral NaCl absorptive process normally inhibiting this process since inhibitors of Ca^{++}/CaM stimulate linked neutral Na and Cl absorption. Attempts to demonstrate a role for protein kinase C in the regulation of rabbit ileal basal Na and Cl absorption have not been successful, although similar techniques have shown a role for protein kinase C in regulating ion transport in other intestinal tissues. Ca^{++}/CaM regulates phosphorylation of rabbit ileal brush border peptides, which indicates that the Ca^{++}/CaM-dependent protein kinases, substrates, and phosphotases are present in this membrane. In whole-cell experiments in which rabbit ileal mucosa is initially incubated with the Ca^{++} ionophore A23187 in the presence or absence of indomethacin, the ionophore caused an increase in phosphorylation of four microvillus membrane peptides with M_r 116,000, 110,000, 53,000, and 32,000. In broken-cell experiments with purified microvillus membranes or brush borders exposed to Ca^{++}/CaM, there was increased

phosphorylation of six peptides with M_r 137,000, 116,000, 77,000, 58,000, 53,000, and 50,000. Ca^{++}/CaM caused a concentration-dependent phosphorylation of these peptides, with the Ca^{++} concentration causing maximal phosphorylation of all being 0.3 μM, except for the M_r 77,000 peptide, in which maximum phosphorylation occurred at 0.8 μM. The M_r 116,000 and 53,000 peptides identified as substrates for the Ca^{++}/CaM kinase had the same size and iso-electric point in broken- and whole-cell studies. Promethazine inhibited all brush border membrane Ca^{++}/CaM-dependent phosphorylations, with the concentration of promethazine required to cause a 50% inhibition being between 2 and 12 μM. The concentration of promethazine required to stimulate Na and Cl absorption (50% effective, 9 μM) was very similar to the concentration inhibiting the Ca^{++}/CaM-dependent phosphorylation. To evaluate whether phosphorylation was involved in regulation of Na and Cl transport, freeze thawing was used to permeabilize brush border membrane vesicles to macromolecules to allow incorporation of ATP plus an ATP-regenerating system. Preliminary data indicate that, although Ca^{++} (1 μM free) plus CaM alone and ATP alone did not affect Na/H exchange in brush border vesicles, the combination inhibited Na/H exchange by ~40%, while substitution of a nonhyrolyzable ATP analogue had no effect. These results indicate that Ca^{++}/CaM-dependent phosphorylation of brush border peptides is involved in the regulation of ileal Na/H exchange.

42. Effect of Changes in Membrane Potential on Intracellular pH of Frog Skin Epithelial Cells K. DREWNOWSKA* and T. U. L. BIBER, *Department of Physiology and Biophysics, Medical College of Virginia, Richmond, Virginia*

Intracellular pH (pH_i) and apical cell membrane potential (V_a) were measured with double-barreled microelectrodes impaled in epithelial cells of frog skin (northern variety of *Rana pipiens*). Isolated frog skins (split skins or whole skins) were mounted in a chamber, equilibrated under open-circuit conditions, and subjected to short periods of voltage-clamping at different levels of transepithelial potential (V_T). Changes in V_T (and therefore in V_a) caused immediate and reversible alterations in pH_i: hyperpolarization of V_a acidified the cell by 0.16 ± 0.01 pH units per 30-mV change in V_a (18 frogs) and depolarization of V_a alkalinized the cell by 0.15 ± 0.01 pH units per 30-mV change in V_a (12 frogs). Exposure to 10^{-3} M DIDS in the apical bath blocked the potential response of pH_i so that intracellular acidification and alkalinization was only, respectively, 0.05 ± 0.03 (seven frogs) and 0.08 ± 0.06 (four frogs) pH units per 30-mV change in V_a. Corresponding treatment with DIDS on the basolateral side had no effect. The removal of HCO_3 from apical and basolateral bath or of Na^+ from the apical bath did not change the response of pH_i to potential changes. As reported previously for short-circuit conditions (Drewnowska and Biber. 1984. *J. Gen. Physiol.* 84:30*a*), the addition of 10^{-4} M amiloride to the apical bath caused also a reversible decrease in pH_i and hyperpolarization of V_a under open-circuit conditions. The amiloride-induced intracellular acidification calculated as ΔpH_i per 30-mV change in V_a was 66 ± 20% (nine frogs) larger than the acidification caused by a voltage pulse. The results indicate that this pH-regulatory mechanism, which acidifies and alkalinizes the cell when V_a is hyperpolarized or depolarized, respectively, is (*a*) inhibited by DIDS, (*b*) located on the apical side, and (*c*) not dependent on the presence of HCO_3 or Na in the apical bath. The results suggest further that the effect of amiloride on pH_i involves, in addition to the above-mentioned mechanism, another pH-regulatory process, possibly the inhibition of an Na^+-H^+ exchange. [Supported by NIH grant 26347.]

43. Observation of Calcium Transients in Isolated Cardiac Myocytes Using Indo-1 W. H. DUBELL,* C. M. PHILLIPS,* and S. R. HOUSER,* *Department of Physiology, Temple University School of Medicine, Philadelphia, Pennsylvania* (Sponsor: Alan R. Freeman)

Indo-1, a calcium-sensitive fluorescent dye (Grynkiewicz et al. 1985. *J. Biol. Chem.* 260:3440), has many properties that make it potentially useful as a means of monitoring and quantifying the rapid calcium transients that cause the twitch in cardiac myocytes. In its unbound form,

when excited with light at 350 nm, indo-1 emits light with a peak at 490 nm. Upon binding to calcium, its emission peak shifts to 420 nm. The ratio of the intensities at each wavelength can potentially be used to quantify free Ca^{2+} during the transient. In the present study, adult feline ventricular myocytes were loaded with indo-1-acetoxymethyl ester (10 μM) for 30 min and washed in Krebs-Henseleit medium with 2 mM Ca^{2+} and 1% albumin. Loaded cells were placed in a 0.75-ml chamber and superfused with normal Tyrode solution (2 mM Ca^{2+}). The chamber was placed on an inverted microscope modified for epifluorescence and photon counting. Experiments were conducted at ambient temperature and the cells were field-stimulated at 0.22 Hz. When a cell was stimulated under these conditions, the fluorescence at 490 nm decreased, whereas that at 420 nm increased. The 420/490 ratio (i.e., the Ca^{2+} transient) rose to a peak in ~1 s and decayed over the next 3.6 s, returning to baseline just ahead of the subsequent stimulus. When the Ca^{2+} was changed to 5 mM, the transient increased in magnitude, with the peak occurring slightly earlier. In addition, the rate of decay increased so that the signal returned to baseline within 3 s of the stimulus. Motion artifact was not ruled out, but the evidence suggests that it is not a major contributor to the signal. This work demonstrates the feasibility of observing calcium transients with indo-1 in adult mammalian cardiac myocytes. [This work was supported by NIH grants HL 33921 and HL 33648. W.H.D. is supported by a fellowship from Berlex Laboratories Inc.]

44. Reconstitution of a Ca^{2+}-dependent Tracheal Chloride Conductance WILLIAM P. DUBINSKY,* *Department of Physiology and Cell Biology, University of Texas Medical School, Houston, Texas* (Sponsor: Stanley G. Schultz)

The tracheal epithelium is a useful model for Cl^- secretion. Secretagogue action on this tissue results in an increase in the apical membrane Cl^- conductance, which is mediated by the second messengers Ca^{2+} and cAMP. We are developing a model to study the regulation of this Cl^- channel in a completely reconstituted system. Bovine trachea are used as the source of a highly enriched apical membrane fraction. The apical membrane is solubilized by detergent extraction and the soluble fraction obtained by ultracentrifrigation. The soluble extract may then be further resolved or otherwise biochemically manipulated. Reconstitution of soluble fractions into artificial phospholipid vesicles is accomplished by the freeze-thaw sonication procedure (Kasahara and Hinkle. 1977. *J. Biol. Chem.* 252:7384). Preliminary studies of the properties of these reconstituted vesicles reveal the following: (*a*) the conductance is inactivated by incubation at 37°C ($t_{1/2}$ = 5 min); (*b*) Ca^{2+} at concentrations >2 × 10^{-7} M activates Cl^- transport; (*c*) a protein fraction can be resolved from the soluble extract by calmodulin affinity chromatography, which is required for maximal activation of the reconstituted transporter; (*d*) the transporter exhibits a selectivity for Cl > Br > gluconate (1:0.2:0.06). [Supported by a research grant from the Cystic Fibrosis Foundation.]

45. The Antipsychotic Pimozide Is a Potent Antagonist of Cardiac Ca^{2+} Channels JOHN J. ENYEART,* VIRENDRA K. SHARMA,* DANIEL J. WILLIFORD,* and SHEY-SHING SHEU, *Department of Pharmacology, University of Rochester Medical Center, Rochester, New York*

Diphenylbutylpiperidine antipsychotics such as pimozide have been suggested to act as Ca^{2+} channel blockers in neurons and smooth muscle based on their ability to inhibit specific [^{3}H]-nitrendipine binding and depolarization-dependent contractions (Gould et al. 1983. *Proc. Natl. Acad. Sci. USA.* 80:5122). To determine the efficacy of these agents as Ca^{2+} antagonists in cardiac cells, we have studied the effect of pimozide on Ca^{2+}-dependent responses in both rat papillary muscles and isolated myocytes. In whole-cell patch voltage-clamp experiments with enzymatically dissociated rat ventricular myocytes, 1 μM pimozide completely blocked Ca^{2+} current activated by 50-mV depolarizing steps from a holding potential of −40 mV. Maximum inhibition was achieved within 2 min and was only partially reversed with repeated washings. Pimozide did not block Na^+ currents in these cells. When intracellular Ca^{2+} in isolated myocytes

was monitored with the fluorescent indicator quin 2, pimozide antagonized the increases elicited by depolarization with 50 mM KCl. Again, 1 μM pimozide was sufficient to block the rise in intracellular Ca^{2+} believed to occur through Ca^{2+} entering voltage-sensitive channels. In conjunction with its blockade of Ca^{2+} channels, pimozide produced a marked inhibition of twitch tension in isolated rat papillary muscles. When muscles were stimulated at 2 Hz, pimozide (1–5 μM) inhibited twitch tension by as much as 50%. These results establish that diphenylbutylpiperadine antipsychotics are relatively potent blockers of "L"-type Ca^{2+} channels in heart. They further suggest that in addition to their neuroleptic activity, these agents may have clinically relevant effects on the cardiovascular system. [Supported by NIH grant HL-33333, an AHA Established Investigator Award, and an AHA/Genesee Valley grant-in-aid.]

46. The Effect of Ca^{2+} and Ca^{2+}-active Drugs on Desensitization to Acetylcholine in *Aplysia* M. L. EVANS* and D. O. CARPENTER, *Wadsworth Laboratories, New York State Department of Health, Albany, New York*

Ca ion is known to affect many cellular processes, including synaptic transmission. Drugs that act on Ca^{2+}-sensitive processes, D-600, SKF 525A, and trifluoperazine, accelerate the rate of onset of desensitization to acetylcholine (ACh) in *Aplysia* neurons. We have investigated the effect of varying the external Ca^{2+} concentration on desensitization. Experiments were performed on voltage-clamped RB cells of desheathed abdominal ganglia. ACh was applied via a rapid local perfusion system. In standard artificial seawater (ASW) containing 10 mM Ca^{2+}, ACh induced (at −50 mV) an inward Na^+ current that rose to a peak within 0.5–1 s and then declined in the continued presence of ACh. The onset of desensitization was described as the sum of two exponential components plus a constant. After 30 min perfusion with 5 mM Ca^{2+} ASW, desensitization was not altered compared with that recorded in normal ASW. After treatment with 0-Ca^{2+} ASW, desensitization was apparently slowed because of elimination of the fast exponential component. In 20 mM Ca^{2+} ASW, desensitization was similar to control values. Elevating the Ca^{2+} concentration to 70 mM increased the peak current amplitude by 50–80% of control. 70 mM Ca^{2+} did not affect the time constant of the fast component, but slowed the time constant of the slow component. The fast component made a greater fractional contribution to the overall response. The phenothiazines promethazine and chlorpromazine accelerate both the fast and slow rates of desensitization at 1–5 μM. A possible mode of action for these drugs is by the inhibition of calmodulin. Chlorpromazine sulfoxide (which has been reported as having no effect on calmodulin) did not affect desensitization at 2 μM, and at 20 μM, it reduced the peak current amplitude by 20% but did not accelerate either component of desensitization. These data indicate that Ca^{2+} may be involved in the onset of desensitization, possibly through an interaction with calmodulin.

47. Direct Measurement of Aberrant Calcium Homeostasis in Duchenne Muscular Dystrophy Fibroblasts EILEEN FINGERMAN,* PAUL L. McNEIL,* JUDITH CAMPISI,* D. LANSING TAYLOR,* and ARTHUR B. PARDEE,* *Department of Biochemistry, University of Massachusetts Medical School, Worcester, Massachusetts; Department of Anatomy, Harvard Medical School, Boston, Massachusetts; Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts; Department of Biological Sciences, Carnegie-Melon University, Pittsburgh, Pennsylvania; and Division of Cell Growth and Regulation, Dana Farber Cancer Institute, Boston, Massachusetts* (Sponsor: John Walsh)

It has been proposed that a membrane defect causing enhanced calcium influx is responsible for the dystrophy in muscle (Rowland. 1980. *Br. Med. Bull.* 36:187). In addition, many of the biochemical and physiological impairments found in Duchenne muscular dystrophy (DMD) can be mimicked in normal cells by experimentally raising the intracellular calcium (Publicover et al. 1978. *Exp. Neurol.* 37:544). In a previous study, we found that DMD fibroblasts were

strikingly more refractory to growth inhibition by calcium- or serum-deficient medium than were normal fibroblasts (Fingerman et al. 1984. *Proc. Natl. Acad. Sci. USA.* 81:7617). Here we provide evidence from direct measurements of intracellular free calcium ($[Ca^{++}]_i$), by aequorin luminescence, that prolonged or transient rises in $[Ca^{++}]_i$ are abnormally suppressed in DMD fibroblasts. Although $[Ca^{++}]_i$ was similar in proliferating normal and DMD fibroblasts, our results demonstrate that DMD fibroblasts regulate $[Ca^{++}]_i$ differently from normal fibroblasts in at least three respects. First, prolonged deprivation of extracellular calcium caused a marked (fivefold) increase in $[Ca^{++}]_i$ in normal cells, whereas DMD cells showed a negligible change. Second, calcium restoration to calcium-deprived cells caused a transient rise in $[Ca^{++}]_i$, which peaked at 1,200 nM in normal cells, compared with a peak value that was three- to fourfold lower (360 nM) in DMD cells. Third, serum restoration to serum-deprived cells also caused a transient rise in $[Ca^{++}]_i$, and this peaked at a value that was approximately twofold lower in the DMD cells. Our results are consistent with the hypothesis that a molecule or process important in both mitogenesis and in maintaining intracellular calcium homeostasis may be more abundant or have an increased affinity for calcium in DMD cells.

48. Ultra-High Resolution of $[Ca^{++}]$ Distribution in Single Cells Revealed by Constrained Inverse Filtering of 3-D Images of Fura-2 Fluorescence KEVIN E. FOGARTY,* DAVID A. WILLIAMS,* and FREDRIC S. FAY, *Department of Physiology, University of Masschusetts Medical Center, Worcester, Massachusetts*

$[Ca^{++}]$ in single living smooth muscle cells has been measured in two dimensions using a digital-imaging microscope equipped for epifluorescence and the highly fluorescent Ca^{++}-sensitive dye, fura-2. In a single image obtained when focused midway through the cell, free Ca^{++} levels over the cytoplasm (137 nM), nucleus (220 nM), and sarcoplasmic reticulum (500 nM) can clearly be distinguished. Uncertainty arises in such images because of the 3-D nature of cellular compartments and the large depth of field of the fluorescence microscope, even using the highest magnification and numerical aperture lenses. Thus, a 2-D image of the 3-D cell structure accesses contributions of Ca^{++} in structures above and below the image plane. We modeled the entire imaging process, including the propagation of detector noise. Assuming cell geometry based upon our biological data and nuclear, SR, and cytoplasmic $[Ca^{++}]$ equal to 269, 425, and 137 nM, respectively, we found that: (*a*) the cytoplasmic $[Ca^{++}]$ measured in an image plane midway through the model image exceeded its true concentration by as much as 55%, because of a mixture of signal from the nucleus and SR, (*b*) nuclear $[Ca^{++}]$ was underestimated by 14%, principally because of mixture with the cytoplasmic signal, and (*c*) SR $[Ca^{++}]$ was underestimated by 24%. In response to this problem, we developed a 3-D image-restoration algorithm based upon a constrained inverse filter together with a Wiener noise filter, to reverse distortion caused by the image-formation process. When it was applied to the model images, we measured $[Ca^{++}]$ with a worst-case error of <1%, and with significantly improved spatial resolution. The restoration algorithm has been applied to 3-D images of fura-2–loaded smooth muscle cells and revealed as yet unresolved details of dye and Ca^{++} distributions, opening the possibility of following $[Ca^{++}]$ changes in organelles during cell response to hormones and neurotransmitters. [Supported in part by grants from the NIH (HL14523) and the Muscular Dystrophy Association.]

49. Calcium and the Occluded State of the Na/K Pump BLISS FORBUSH III,* with the technical assistance of GRACE JONES and JOHN T. BARBERIA, *Department of Physiology, Yale University School of Medicine, New Haven, Connecticut* (Sponsor: Mark Haas)

From a study of the effects of Ca on phosphorylation of Na,K-ATPase, Post (1984. *Biophys. J.* 45:78*a*) has proposed that Ca ions may be tightly bound at K transport sites in the "occluded state" of the pump cycle. We report that upon incubation of ^{45}Ca with Na,K-ATPase in the

presence of 3 mM Mg, ^{45}Ca is indeed tightly bound to the Na,K-ATPase, in a manner consistent with the hypothesis that Ca binds to the transport site. Approximately one ^{45}Ca ion is bound per active Na,K-ATPase unit, with an affinity of ~0.17 mM. The spontaneous rate of ^{45}Ca dissociation from this site is 2.2 s^{-1} at 20°C in the absence of cations; this is accelerated (to 95 s^{-1}) by ATP (or ADP) binding at a low-affinity site. Surprisingly, in contrast to ^{86}Rb release, ^{45}Ca release is *slowed* (to 1.7 s^{-1}) upon phosphorylation with MgP_i, which indicates that the occluded state may not release Ca toward the extracellular surface of the Na/K pump. Rb blocks ^{45}Ca binding with high affinity ($K_{1/2}$ = 0.02 mM at 0.167 mM ^{45}Ca); thus, it is unlikely that there is significant Ca binding to these sites under physiological conditions. We have found the following actions of Ca on the release of ^{86}Rb from the occluded state: (*a*) in the presence of ATP, Ca behaves almost exactly as Mg does, stimulating the rate of deocclusion in the range where CaATP or MgATP are formed, and inhibiting at high concentrations ($K_{1/2}$ = 7 mM for Ca, 10 mM for Mg); (*b*) with P_i, Ca competes with Mg ($K_{1/2}$ = 0.11 mM at 1 mM Mg) and prevents ^{86}Rb release, probably by blocking phosphorylation (see Post, *ibid*). Finally, in a related study, we have been unable to detect tight binding of ^{86}Rb to Ca-ATPase isolated from sarcoplasmic reticulum. [Supported by GM31782.]

50. Abnormalities of Ca^{++} Transport in Synaptosomes from Uremic Rat Brain: Role of Parathyroid Hormone COSMO L. FRASER* and ALLEN I. ARIEFF,* *Department of Medicine, VA Medical Center and University of California School of Medicine, San Francisco, California*

Previous studies from this laboratory have demonstrated that the Ca^{++} content of cerebral cortex in vivo is increased in uremia, and that this increase in Ca^{++} content is parathyroid hormone (PTH) dependent (Cooper et al. 1978. *J. Clin. Invest.* 61:1448). More recently, we have shown that in synaptosomes isolated from uremic rat brain, there is increased transport of Ca^{++} by both ATP-dependent Ca^{++} transport and Na^+/Ca^{++} exchange mechanisms (Fraser et al. 1985. *J. Clin. Invest.* 76:1789). The current study was designed to investigate the role that PTH may play in the abnormalities of Ca^{++} transport observed in synaptosomes from uremic rat brain cerebral cortex. Synaptosomes were extracted from rat brain by homogenization and differential centrifugation as a modification of the method of Gray and Whittaker (1962. *Biochem. J.* 90:293), and Ca^{++} transport by both ATP stimulation and Na^+/Ca^{++} exchange was studied in vitro. In uremic rats (urea = 80 mM), Ca^{++} transport was increased by 26 and 21% by both ATP-dependent and Na^+/Ca^{++} exchange, respectively. However, when uremic rats had been previously subjected to parathyroidectomy (PTX-U), Ca^{++} transport fell from the uremic control values to normal control levels. However, when PTX-U rats were given intraperitoneal injections of PTH, Ca^{++} transport was again increased to levels observed in intact uremic animals. In normal rats, parathyroidectomy resulted in decreased Ca^{++} transport by values of 30 and 24% by Na^+/Ca^{++} exchange and ATP-dependent transport mechanisms, respectively. As was observed in uremia, PTH administration to parathyroidectomized rats increased Ca^{++} transport back to control values. These studies show that in synaptosomes from uremic rat brain: (*a*) Ca^{++} transport by both ATP-dependent and Na^+/Ca^{++} exchange mechanisms is increased; (*b*) the increased Ca^{++} transport observed is largely PTH dependent; however, the uremic environment appears to play an important role in enhancing the action of PTH. [Supported by the Research Service of the Veterans Administration Medical Center, San Francisco, CA.]

51. Interaction of Tetrandrine with Ca^{2+} Channels in Cardiac Sarcolemmal Membrane Vesicles M. L. GARCIA,* V. F. KING,* Y. LAM,* J. X. PAN,* and G. J. KACZOROWSKI,* *Merck Institute for Therapeutic Research, Rahway, New Jersey* (Sponsor: J. P. Reuben)

Tetrandrine is a dibenzyl isoquinoline alkaloid isolated from *Stephania tetrandra*, which is used in China for the treatment of hypertension. Several recent pharmacological studies suggest

that this compound may function as a Ca^{2+} entry blocker but its precise mechanism of action remains unknown. To determine what effect, if any, tetrandrine has on voltage-dependent Ca^{2+} channels in heart, the interaction between this agent and the Ca^{2+} entry blocker receptor complex of purified porcine cardiac sarcolemmal membrane vesicles was characterized. We have previously shown (Garcia et al. 1986. *J. Biol. Chem.* In press) that three distinct high-affinity binding sites exist for the three structural classes of Ca^{2+} entry blockers in this membrane preparation, that these sites are allosterically coupled, and that they are functionally associated with Ca^{2+} channels. Tetrandrine was found to produce a concentration-dependent inhibition of both D-600 and diltiazem binding at 25°C (K_i's of 0.56 and 0.57 μM with partial and complete inhibition, respectively). Strikingly, tetrandrine has little effect on nitrendipine binding at 25°C, but produced a concentration-dependent stimulation of binding at 37°C. Scatchard analyses of D-600, diltiazem, and nitrendipine binding at equilibrium in the presence of tetrandrine indicate that this compound lowers the maximal number of aromatic alkyl amine receptors, decreases the affinity of the benzothiazepine receptors, and increases the affinity of dihydropyridine receptors (at 37°C). This profile is similar to that of diltiazem itself and suggests that tetrandrine, even though it is structurally dissimilar from diltiazem, interacts directly at the benzothiazepine receptor and allosterically modulates ligand binding at the other two Ca^{2+} entry blocker receptor sites. These results predict that tetrandrine would be an inhibitor of voltage-dependent Ca^{2+} channels and provide a mechanistic basis for its known pharmacological activity.

52. Studies on the Increase of Cytosolic Free Ca^{++} Induced by Epidermal Growth Factor, Serum, and Nucleotides in Individual A431 Cells F. A. GONZALEZ,* D. J. GROSS,* L. A. HEPPEL,* and W. W. WEBB,* *Section of Biochemistry, Molecular and Cell Biology and School of Applied and Engineering Physics, Cornell University, Ithaca, New York* (Sponsor: G. W. G. Sharp)

Using fura-2 and quantitative digital video fluorescence microscopy of individual cells, we have confirmed the fluorimeter study of Moolenaar et al. (1986. *J. Biol. Chem.* 261:279) that epidermal growth factor (EGF) caused a two- to fourfold transient rise in $[Ca^{++}]_i$ of A431 cells dependent on external Ca^{++}. We further noted that different cells in a field of view responded with different "lag" times. We agree that 1–2% fetal calf serum causes a similar rise that does not require external Ca^{++}, but with some lots only 0.08% serum was needed. In addition, with 1 mM Ca^{++}, cells responded to 25 μM ATP or UTP, but showed only a small, variable rise in $[Ca^{++}]_i$ with 100 μM GTP and no rise with 100 μM ITP or CTP. In further trials, agonist was added at 40 s, removed by washing at 100 s, followed by agonist at 140 s. (1) With 1 mM external Ca^{2+}: (*a*) 50 μM ATP caused a rapid rise and a sustained response; a second addition of ATP caused little or no response; (*b*) 0.4% serum caused a large response, which was attenuated upon the second serum addition; (*c*) 100 μM AMPPNP caused no response and no interference with the second addition of 50 μM ATP; (*d*) EGF (100 ng/ml) gave a response, as did a subsequent addition of 50 μM ATP. (2) In "Ca^{++}-free" medium with 100 μM EGTA: (*a*) 50 μM ATP caused a slow response, then a drop to baseline, and no response to a second addition; (*b*) serum (0.4%) caused a strong response upon the first addition, but not upon the second addition; (*c*) ATP (50 μM) following serum caused a partial response; (*d*) there was a strong response to the first addition of 50 μM UTP; the second addition was negative; (*e*) there was a strong response to 50 μM ATP, after which the response to 50 μM UTP was blocked. These results and related studies by others will be discussed. [Supported by grants from Biotechnology Program (Cornell), NIH, NSF, and ONR.]

53. Multiple Types of Calcium Channels in Acutely Exposed Neurons from the Adult Guinea Pig Hippocampus RICK GRAY* and DAN JOHNSTON, *Program in Neuroscience, Baylor College of Medicine, Houston, Texas*

We have been studying macroscopic calcium currents and their modulation by beta-adrener-

gic agonists in exposed neurons from guinea pig hippocampal slices (Gray and Johnston. 1985. *Biophys. J.* 47:66*a*). During these investigations, we have observed that inward currents were detectable only during step commands from −100 mV to potentials more positive than about −35 mV. Also, the whole-cell calcium currents appeared to inactivate partially, even with 10 mM EGTA inside the cell and with Ba as the charge carrier, which suggests that some component of the current is transient. In light of these observations and the recent report of multiple types of Ca channels with different gating characteristics in cultured neurons (Nowycky et al. 1985. *Nature* [*Lond.*]. 316:440), we have begun to measure single channel currents in our system. Acutely exposed neurons were prepared as described elsewhere (Gray and Johnston. 1985. *J. Neurophysiol.* 54:134) and were patch-clamped in the cell-attached configuration with 110 mM $BaCl_2$ in the pipette and 140 mM K-aspartate in the bath. Patches were held at −90 mV and depolarizing command pulses were applied. The amplitudes of unitary currents at different command potentials were used to construct single channel *I-V* plots. We have observed single channels with three slope conductances: 7–8, 13–15, and 25–27 pS. The 7–8-pS channel appeared more transient in nature than the others, with openings clustered near the onset of the step. Future experiments will be directed at finding which of these channels are modulated by beta-adrenergic receptors. [Supported by NS11535, NS15772, and AFOSR 85-0178.]

54. Serosal Membrane Conductances and the Effect of Reducing Bath Na in Guinea Pig Gallbladder

PAMELA J. GUNTER-SMITH, *Physiology Department, Armed Forces Radiobiology Research Institute, Bethesda, Maryland*

The effect of serosal bath ion substitutions on the membrane potential (V_b) and fractional resistance (f_{Rb}) of the serosal membrane of guinea pig gallbladder epithelial cells was determined. Increasing the bath K concentration (K_s) from 5 to 50 mM (cation substitutions were by equimolar replacement of *N*-methyl-D-glucamine) depolarized V_b and decreased f_{Rb}. Decreasing Na from 130 to 13 mM (low Na_s) depolarized V_b and increased f_{Rb}. Substitution of Cl by SO_4 or HCO_3 by HEPES had little effect on V_b or f_{Rb}. The effects of quinidine (10^{-4} M), added to the serosal solution, were qualitatively similar to those of low Na_s. In addition, the response of the tissue to quinidine was reduced in low Na_s. The Na transport inhibitors amiloride (10^{-3} M) and bumetanide (10^{-5} M) had no significant effect on V_b and f_{Rb}. However, in the presence of amiloride, V_b tended to hyperpolarize and f_{Rb} to increase. The effect of low Na_s and quinidine on the response of the tissue to elevating K_s were similar: the magnitude of the high K_s depolarization decreased. Taken together, the results suggest that the serosal membrane is primarily K selective. The response to low Na_s cannot be reconciled with the presence of a serosal membrane conductance for this ion, but rather appears to be related to a secondary decrease in serosal membrane K conductance via a quinidine-sensitive process or pathway. These results are similar to those obtained in other epithelia in which it has been suggested that the effects of low Na_s are secondary to a reduction in transcellular Na transport via an inhibition of quinidine-sensitive Na-Ca exchange and increased intracellular Ca.

55. Kinetics of a Calcium-activated Potassium Current in Rat Sympathetic Neurons Studied with the Photosensitive Calcium Chelator Nitr-5

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We have used the photosensitive calcium chelator nitr-5 (Adams et al., this meeting; we thank R. Y. Tsien for providing nitr-5) to study Ca-sensitive membrane currents in neonatal rat superior cervical ganglion cells. Outward K currents were recorded at 20°C using the whole-cell patch-clamp technique. Nitr-5 (2–10 mM), complexed with calcium, was included in the KCl solution filling the patch pipettes so that the concentrations of nitr-5 and Ca inside the cell presumably reflected those in the pipette. Light flashes (1 ms duration) should then cause "jumps" in the intracellular Ca concentration. Flashes induced outward currents that were

usually maintained for at least 200 ms. In cells exposed to nitr-5, even without added Ca, a flash-induced current was observed. The current amplitude at a membrane potential of 40 mV was linearly related to the flash intensity, which suggests that more than one Ca molecule may not be necessary for channel opening. Current activation followed an exponential time course, the time constant varying with the amplitude of the Ca jump and with the membrane potential. With either 2 or 10 mM nitr-5, 75% complexed with Ca, the time constant varied from 38 ± 10 ms (mean ± SEM; $n = 4$), with a concentration jump estimated at 40 nM, to 5.4 ± 0.7 ms ($n = 12$), with an estimated jump of 1,600 nM. Activation was faster at depolarized potentials. The current induced in one cell by an estimated Ca jump of 152 nM relaxed with a time constant of ~18 ms at 20 mV, compared with 11 ms at 60 mV. The concentration and voltage dependence of the activation kinetics imply that they are not limited by the rate of Ca release from nitr-5. The data provide direct confirmation that K channels can be activated within a few milliseconds after increases in the intracellular Ca concentration. [Supported by GM-29836, MRC, British Heart Foundation.]

56. Calcium Handling by Normal and Diseased Human Myocardium JUDITH K. GWATHMEY,* LINDA COPELAS,* WILLIAM GROSSMAN,* and JAMES P. MORGAN,* *Department of Medicine, Harvard Medical School, Beth Israel Hospital, Boston, Massachusetts* (Sponsor: K. G. Morgan)

We chemically loaded aequorin, a bioluminescent Ca^{++} indicator, into trabecular strips obtained from patients with dilated cardiomyopathy ($n = 19$), hypertrophic cardiomyopathy ($n = 2$), and control samples ($n = 4$) obtained from organ donors. Trabeculae carneae of ≤1.2 mm diam were selected, perfused in a physiologic salt solution, and maintained at 30°C in a special light-collecting apparatus. Muscles were stretched to L_{max}. Action potentials were done on the same or similar muscles using standard microelectrode techniques. Gwathmey et al. (1985. *Circulation.* 72:484) reported that the calcium transient from patients with dilated cardiomyopathy consisted of two distinct components (L_1 and L_2), whereas in control hearts (C), the light consisted of a single component (L_1). In order to test whether L_2 in myopathic muscle reflected a decreased capacity to handle increases in cytosolic Ca^{++}, calcium dose-response curves were performed. In the hypertrophic and dilated cardiomyopathic hearts, there was an increase in the amplitude of L_2 relative to L_1. In nine myopathic muscles, there was an increase in the end-diastolic levels of light and tension; this did not occur in controls. To determine the sources of Ca^{++} responsible for L_1 and L_2, we studied the effects of ryanodine, an alkaloid that blocks release of Ca^{++} from the sarcoplasmic reticulum (SR). In control muscle, ryanodine abolished the monophasic Ca^{++} transient. In myopathic muscle, a low dose of ryanodine abolished L_1 without significantly affecting the amplitude of L_2. A maximally effective dose of ryanodine did not completely suppress the Ca^{++} transient, which suggests that this residual component of L_2 arises from some source other than SR. The addition of verapamil, a Ca^{++} channel blocker, suppressed the light remaining after ryanodine, which indicates that a significant component of L_2 reflects Ca^{++} entry through voltage-dependent sarcolemmal channels. Action potential durations from myopathic strips were prolonged compared with controls suggestive of a prolonged slow inward current. Our data support the idea that L_1 reflects release from the SR and that L_2 is a combination of SR and slow inward current. [Supported by NIH grants HL31117, HL01611, HL36797, and HL07374.]

57. Properties of Na/K Pump in Human Red Cells with an Increased Number of Pump Sites JOSE A. HALPERIN,* CARLO BRUGNARA,* JOANNE S. INGWALL,* and DANIEL C. TOSTESON, *Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts*

We describe the kinetic properties of the Na/K pump of the red cells of a patient (MAJ cells), which have (relative to control values) a 10–20-fold-higher number of ouabain-binding sites, 4-

fold-lower intracellular Na (Na_i) and similar total cations, ATP, ADP, and P_i content. We have found that the maximum turnover number, apparent affinity for Na_i, and other kinetic properties of the pumps in MAJ cells and normal control cells are similar. Despite the high pump density (maximum ouabain-sensitive Na efflux and lactate production of 60 and 12 in MAJ compared with 6 and 2 mmol/liter cell·h in normal controls), the ouabain-sensitive Na efflux and lactate production in fresh MAJ cells were similar to values observed in control cells. The reduction in Na_i in fresh MAJ cells reduces Na pump efflux to equal the ouabain-insensitive Na influx, which is similar in MAJ cells and normal control cells. We observed that the ouabain-sensitive Na efflux was not stimulated, whereas the ouabain-sensitive K influx was stimulated by external K (K_o). This result is not due to a peculiarity of MAJ cells, since the ouabain-sensitive Na efflux of normal cells containing low Na_i (≈2 mmol/liter cell) also fails to respond to changes in extracellular K. We have further found that at this Na_i, the ouabain-sensitive Na efflux through the Na_i-Na_o exchange in K-free medium equals the Na efflux through the Na_i-K_o exchange at high K. The inhibition of the Na_i-Na_o exchange and the stimulation of the Na_i-K_o exchange by external K maintains a constant ouabain-sensitive Na efflux at each K_o concentration.

58. Role of Extracellular Calcium in Contraction of Smooth Muscle in the Upper Gastrointestinal Tract of the Cat C. HILLEMEIER,* J. BEHAR,* J. MARSHALL, and P. BIANCANI,* *Departments of Pediatrics and Internal Medicine, Rhode Island Hospital and Brown University School of Medicine, Providence, Rhode Island*

Single cells were isolated from the circular muscle layers of the esophagus, lower esophageal sphincter (LES), inner muscle layers of the fundus, and circular muscle layers of the antrum, pylorus, and duodenum. The mucosa and longitudinal muscle were removed and the tissue was digested in collagenase to isolate single muscle cells. After digestion, the cells were rinsed and then harvested in collagenase-free solution. Dose-response curves were obtained to acetylcholine (ACh) in the presence of physiological amounts of Ca^{++} (2 mM), after the addition of the calcium channel blocker methoxyverapamil (D-600), in Ca^{++}-free solution containing 2 mM EGTA, and in calcium-free solution containing strontium (Sr^{+3}), which depletes intracellular calcium stores. Cells from all regions exhibited similar resting length (76.9–90.1 μm) and ACh dose-response relationships contracting maximally at 10^{-10}–10^{-9} M ACh. Incubation in calcium-free solution and in D-600 completely abolished contraction in esophageal and duodenal muscle cells, but did not affect dose-response curves and percent maximal extraction of cells from the LES, fundus, and pylorus. Contraction of cells from the antrum was reduced 67.1% in calcium-free solution and 59.1% in D-600. Conversely, Sr^{+3} abolished contraction in cells from the LES, fundus, and pylorus. We conclude that smooth muscle cells from LES, fundus, and pylorus use intracellular Ca^{++} to contract in response to ACh, whereas the body of the esophagus, the duodenum, and to some extent the antrum need extracellular Ca^{++} in order to contract in response to ACh. It is possible that the ability to use intracellular Ca^{++} may be a characteristic of muscles that, like the LES, pylorus, and fundus, exhibit tonic activity.

59. Calcium Channel Blockers Interact with the α_2-Adrenergic Receptor in the Basolateral Membrane of Rabbit Ileum FADIA R. HOMAIDAN,* MARWAN E. EL-SABBAN,* JOAN WICKS,* SHEILA CUSOLITO,* MARK DONOWITZ, and GEOFFREY W. G. SHARP, *College of Veterinary Medicine, Cornell University, Ithaca, New York, and Gastroenterology Unit, New England Medical Center Hospital, Tufts University, Boston, Massachusetts*

An interaction between the α_2-adrenergic receptor and Ca^{++} channel blockers was demonstrated in rabbit ileum. The effects of clonidine, an α_2-adrenergic agonist, on active electrolyte transport, using the Ussing chamber/voltage-clamp technique, were evaluated in the presence and absence of several classes of Ca^{++} channel blockers. In addition, the effects of these Ca^{++} channel blockers on the binding of [^{3}H]clonidine and [^{3}H]yohimbine (an α_2-adrenergic antago-

nist) to an enriched preparation of basolateral membranes of ileal epithelial cells were studied. Ca^{++} channel antagonists and clonidine both decreased short-circuit current (I_{sc}) and increased Na and Cl absorption in a dose-dependent manner. However, when verapamil, diltiazem, and cadmium were added to clonidine-treated tissues, an inhibition of the effects of clonidine on transport was observed. These agents also inhibited specific [^{3}H]clonidine or [^{3}H]yohimbine binding to ileal basolateral membranes. The concentration dependence of the Ca channel blockers to inhibit the transport effects of clonidine were similar to the concentration dependence to inhibit specific α_2-receptor binding. In contrast, nifedipine had no effect on either the clonidine response on transport or α_2-receptor binding. We suggest that an interaction exists between the three classes of calcium channel blockers and the α_2-receptor. The data can be explained by either the existence of a close spatial relationship between the calcium channel and the α_2-receptor, such that the binding of the calcium channel blockers alters the conformation of the α_2-receptor, and affects binding to the receptor, or by a nonspecific effect of verapamil, diltiazem, and cadmium exerted directly on the α_2-receptor.

60. Effect of Anisosmotic Medium on Cell Volume, Transmembrane Potential, and Intracellular K^+ Acti .ıy in Mouse Hepatocytes LARRY D. HOWARD* and ROBERT WONDERGEM, *Department of Physiology, Quillen-Dishner College of Medicine, East Tennessee State University, Johnson City, Tennessee*

We have determined that mouse hepatocytes maintain their cell volume when exposed to anisosmotic medium, and we have begun to explore cellular mechanisms of this regulation. Mouse hepatocytes in primary monolayer culture (4 h) were exposed for 10 min at 37°C to anisosmotic medium of altered NaCl concentration. Cell water was measured by subtracting the extracellular water determined by the distribution volume for [^{14}C]polyethyleneglycol from the total water determined gravimetrically. Hepatocytes maintained constant relative cell volume (experimental vol/control vol) as a function of external medium relative osmolality (control mosmol/experimental mosmol) ranging from 0.8 to 1.5. In contrast, the relative cell volume fit a predicted Boyle-Van't Hoff relationship when the experiment was repeated at 4°C. Mouse liver slices were used for electrophysiologic studies, in which hepatocyte transmembrane potential (V_m) and intracellular K^+ activity (a^i_K) were recorded continuously by open-tip and liquid ion-exchanger ion-sensitive glass microelectrodes, respectively. Liver slices were superfused with control and then with anisosmotic medium of altered NaCl concentration. V_m increased in response to hypoosmotic medium and decreased with hyperosmotic medium, and ln [10·(experimental V_m/control V_m)] was a linear function of relative osmolality (control mosmol/experimental mosmol) in the range 0.8–1.5. The a^i_K did not change when medium osmolality was decreased 40–70 mosmol from a control of 280 mosmol. Similar hypoosmotic stress in the presence of either external 60 mM K^+ or 1 mM quinine HCl or at 27°C resulted in no change in V_m compared with a 20-mV increase in V_m in response to hypoosmotic stress without the added agents or at 37°C. $BaCl_2$ (1 mM) also inhibited the increase in V_m with hypoosmotic stress but only by 6 mV. We conclude that mouse hepatocytes maintain their volume and a^i_K in response to anisosmotic medium; however, V_m behaves as an osmometer under these conditions. Since increases in V_m by hypoosmotic stress were abolished by conditions or agents that inhibit K^+ conductance, hepatocyte volume regulation may comprise changes in hepatocyte membrane K^+ permeability and conductance during osmotic stress. [Supported by The Kroc Foundation.]

61. Na-coupled Glycine Uptake by Ascites Tumor Cells Is Accompanied by an Increase in Membrane Channel Activity RANDALL L. HUDSON* and STANLEY G. SCHULTZ, *Department of Physiology and Cell Biology, University of Texas Medical School, Houston, Texas*

The addition of *l*-glycine (10 mM) to the solution bathing Ehrlich ascites tumor cells patch-clamped in the cell-attached configuration results in a marked increase in single channel activity

of the plasma membrane. Results obtained with the whole-cell recording technique indicate that: (*a*) the addition of glycine to the bathing solution brings about an initial depolarization of the electrical potential difference across the plasma membrane that is followed by a partial repolarization; and (*b*) bathing the cells with 150 mM KCl or 150 mM RbCl abolishes the transmembrane electrical potential difference. These results closely parallel those previously reported for Na-coupled sugar and amino acid uptake by *Necturus* small intestinal absorptive cells (Gunter-Smith et al. *J. Membr. Biol.* 66:25; Grasset et al. 1983. *J. Membr. Biol.* 71:98; Hudson and Schultz. 1984. *Science.* 224:1237) and are consistent with the notion that an increase in Na-K pump activity and/or cell swelling somehow "triggers" a parallel increase in plasma membrane K conductance. [Supported by NIH grant AM-26990.]

62. Growth Factor-induced Transient Acidification Is Caused by Increased Intracellular Ca^{++} H. E. IVES* and T. O. DANIEL,* *Division of Nephrology, Cardiovascular Research Institute and Howard Hughes Medical Institute, University of California, San Francisco, California* (Sponsor: R. J. Alpern)

Exposure of fibroblasts to a variety of growth factors results in rapid changes in intracellular pH and [Ca^{++}]. We studied the pattern of these ionic responses after exposure of 3T3 cells to platelet-derived growth factor (PDGF), phorbol myristate acetate (PMA), bradykinin, and NH_4Cl. After exposure, intracellular pH and Ca^{++} were measured using the fluorescent dyes (2′,7′)-bis(carboxyethyl)-(5,6)-carboxyfluorescein and fura-2. Three patterns of intracellular pH change were observed. PDGF caused a rapid amiloride-resistant acidification (<2 min) of 0.03 ± 0.01 pH units, followed by a slower (5–10 min) amiloride-sensitive alkalinization of 0.11 ± 0.02 units above the resting pH of 6.9. Bradykinin caused rapid acidification and subsequent correction of pH without the slower alkalinization. PMA caused only alkalinization without the early acidification. The intracellular Ca^{++} responses to the three agents were also different. PDGF caused a rapid (<3 min) increase in intracellular Ca^{++} from a resting level of 80 ± 10 nM to 170 ± 30 nM and back to 100 ± 20 nM within 5 min of stimulation. Bradykinin increased cytoplasmic Ca^{++} by similar amounts but over a more rapid (1 min) time course. PMA caused no change in intracellular Ca^{++}. None of the agents caused Ca^{++} transients when intracellular Ca^{++} was buffered by loading with 120 μM quin-2 in a 0 Ca^{++}, 5 mM EGTA solution. In Ca^{++}-buffered cells, bradykinin and PDGF failed to induce cellular acidification. However, stimulation of the Na^+/H^+ exchanger by PMA and PDGF was unaltered by Ca^{++}-clamping. Neither NH_4Cl exposure nor washout induced any change in intracellular Ca^{++}. Thus, the transient acidification seen with PDGF and other growth factors appears to be the result of increased intracellular Ca^{++}. Agents like PMA, which do not raise intracellular Ca^{++}, do not cause acidification. Stimulation of the Na^+/H^+ exchanger is independent of intracellular Ca^{++} increases. [Supported in part by NIH grant AM-34127.]

63. Calcium Gradients Measured with a Vibrating Calcium-selective Electrode LIONEL F. JAFFE and SIMON LEVY,* *Marine Biological Laboratory, Woods Hole, Massachusetts, and Department of Physiology, Boston University Medical School, Boston, Massachusetts*

It has proposed that ultrasensitive, vibrating, extracellular, ion-selective microelectrodes could be developed to measure specific ion gradients and thus infer specific local ion currents into or out of cells or tissues (Jaffe. 1986. *Ciba Symp.* 122). We now report such experiments using calcium-selective electrodes. We used an artificial calcium source consisting of a blunt, 15-μm-diam pipette filled with 300 mM $CaCl_2$ (plus 0.5% agar) immersed in 300 mM $MgCl_2$ (plus 0.3 mM $CaCl_2$). We explored free calcium gradients around this source using a 12-μm-diam Ca-selective electrode vibrating over 12 μm at ~1 Hz. The Ca-selective electrode was filled with a liquid sensor that was based on the neutral carrier ligand of Oehme et al. (1976.

Chimia. 30:203) and had a resistance of a few gigohms. Its output was fed into a lock-in amplifier. In this way, gradients could be measured from 50 μm to 2 mm away from the source. The measured size of these gradients fell off with distance from the source in a way that approximates steady state theory. The smallest gradients measured in this first effort indicated a 0.2% difference in free calcium concentration over the 12-μm amplitude of vibration in a background concentration of ~0.3 mM calcium. Such a difference represents a local calcium current of ~7 pmol/cm^2·s. We believe that this sensitivity can be considerably improved and expect to report further progress at this meeting. [Supported by the National Vibrating Probe Facility.]

64. Ionomycin Effect on the Hydroosmotic Response to Vasopressin (VP) and cAMP in Rabbit Cortical Collecting Tubule (CCT) S. M. JONES,* G. FRINDT,* and E. E. WINDHAGER* *Cornell University Medical College, New York* (Sponsor: L. G. Palmer)

Several studies suggest that changes in intracellular calcium ion concentration can modulate the magnitude of the VP- and cAMP-dependent hydraulic conductivity of CCTs. In these studies, a reduction in the peritubular Na concentration, the addition of quinidine, or the elevation of the calcium concentration in the basolateral bathing solution have been used as maneuvers to elevate cytoplasmic calcium ion levels. Although the hydroosmotic response of CCTs to VP and cAMP was consistently reduced by these experimental conditions, the question whether cytosolic calcium was actually increased was never answered directly. Since measurements of intracellular calcium ion concentrations in a perfused, transporting CCT are presently not available, experiments were performed using the calcium ionophore ionomycin as a tool to raise intracellular calcium. The effect of ionomycin on the development and on the maintenance of a fully developed response to VP (20 μU/ml) or 8-[4-chlorophenylthio]-cyclic AMP (10^{-4} M) was studied. To examine the initial phase of the hormone response, a series of experiments was performed in which ionomycin and VP were added to the bathing fluid simultaneously. The VP-stimulated L_p (cm·s^{-1}·atm^{-1} × 10^{-7}) in eight control tubules was 275 ± 27; in three tubules tested with 10^{-7} M ionomycin, L_p was 91 ± 42 ($p < 0.001$); in five tubules exposed to 10^{-6} M ionomycin, L_p averaged 101 ± 11 ($p < 0.001$). In other experiments, the maintenance phase of the VP response (after the initial 30 min) was examined; ionomycin (10^{-6} M) added to the bath for 15 min reversibly reduced the hydroosmotic response by 42 ± 8% ($n = 10$; $p < 0.001$, paired t test). The same dose of ionomycin, when added for 30 min at 30 min after the addition of cAMP, reduced the nucleotide response by 35 ± 5% ($n = 6$; $p < 0.001$, paired t test) at 50 min after the administration of the ionophore. These data support the view that increases in cytosolic calcium ion concentration inhibit the VP and cAMP response of mammalian cortical collecting tubules. [Supported by NIH R01 AM11489, the N. Y. Heart Association, and BRSG RR05396.]

65. Identification of a High-Affinity Calmodulin Acceptor Protein from the *Electrophorus electricus* Electric Organ MARCIA A. KAETZEL* and JOHN R. DEDMAN,* *Department of Physiology and Cell Biology, University of Texas Medical School, Houston, Texas* (Sponsor: Stanley G. Schultz)

Mediation of the calcium signal through calmodulin has been suggested by the presence of calmodulin-dependent kinase, calcineurin, fodrin, and phosphodiesterase in synaptic junctions. To further elucidate the role of calmodulin, we have investigated calmodulin acceptor proteins (CAPs) in the higher differentiated eel electric organ. This tissue is developmentally derived from muscle and contains the highest known concentration of calmodulin and its mRNA. The major calmodulin acceptor protein in the electric organ possesses an exceptionally high affinity for calmodulin. This 55-kD peptide has been purified to homogeneity by ion exchange, calmodulin–Sepharose 4B affinity chromatography, and electrophoretic elution from preparative gels. A smaller 47-kD peptide also exhibits high-affinity interaction with calmodulin.

Antibody against each peptide was raised in sheep and was affinity-purified. Comparison of limited protease digestions and epitope recognition suggests that the 47-kD CAP is derived from the 55-kD CAP during isolation. Although the 55-kD CAP is a major protein of the electric organ, only trace amounts are present in muscle. It is not detectable in nonexcitable tissue such as liver. Characterization of this 55-kD high-affinity calmodulin acceptor protein should yield further insight into the role of calcium-calmodulin in excitable tissues. [Supported by GM 39232.]

66. Swelling-activated K Efflux in Human Erythrocytes: Dependence on External K D. KAJI,* with the technical assistance of J. AMBLARD,* *Veterans Administration Medical Center, Bronx, New York, and Mount Sinai School of Medicine, New York* (Sponsor: T. J. McManus)

The swelling of human erythrocytes activates a Cl-dependent, Na-independent K influx pathway (1985. *J. Gen. Physiol.* 86:40*a*). However, it has remained unclear whether this K influx represents a strictly obligatory K-K exchange or whether swelling-activated K transport is capable of net K transport. To distinguish between these possibilities, K efflux from swollen cells was measured in hypotonic Cl or NO_3 media with and without external Rb. Simultaneously, unidirectional K influx was measured with ^{86}Rb. We obtained the following results. (*a*) In media without Rb, K efflux from swollen cells was significantly enhanced as compared with nonswollen cells, and this enhancement was Cl dependent, which suggests that the swelling-activated K transport pathway is capable of net K efflux. (*b*) The addition of external Rb led to further enhancement of Cl-dependent K efflux from swollen cells, which is probably due to K-Rb exchange. (*c*) The K efflux was a saturable function of external K, with a $K_{1/2}$ of 24 mM. (*d*) A comparison of simultaneous K influx and efflux allowed the calculation of net K fluxes. Net efflux progressively decreased as external K was raised. At 40 mM K_o, no net flux was seen. Above this value, a net K influx was observed that increased with increasing external K. The swelling-activated K transport in human erythrocytes can mediate net K influx or efflux, depending on the external K or the K_i/K_o ratio.

67. The Dependence on Cell Na (Na_i) of Chloride-dependent K Influx in Human Erythrocytes: Effect of Cell Swelling and *N*-Ethylmaleimide (NEM) D. KAJI,* with the technical assistance of J. AMBLARD,* *Veterans Administration Medical Center, Bronx, New York, and Mount Sinai School of Medicine, New York* (Sponsor: T. J. McManus)

A component of the Cl-dependent K influx in human erythrocytes is observed in media without Na (Na_o) and has been proposed to represent the KCl cotransport pathway. However, the dependence of this Na_o-independent pathway on the intracellular Na (Na_i) has not been examined. This study examined the effect of removing cell Na on (*a*) the Na_o-independent, Cl-dependent K influx in nonswollen cells, (*b*) the swelling-activated K influx, and (*c*) the NEM-stimulated K influx. Cell electrolytes were altered by the nystatin method (Na replaced by K) and influx was measured with ^{86}Rb in anion-equilibrated cells in media containing Cl or NO_3 salts of *N*-methylglucamine and 30 mM K. In the total absence of internal and external Na, the Cl-dependent K influx in nonswollen cells was abolished, which suggests that the Na_o-independent component represents a partial reaction (K-K exchange mode) of the putative NaKCl cotransport pathway. In contrast, in the presence of either cell swelling or NEM, Cl-dependent K influx is independent of intracellular as well as external Na, which suggests that swelling and NEM may activate a putative KCl cotransport pathway.

68. Phloretin Inhibits and Activates Separate Modes of Net Sulfate Transport in Red Blood Cell Ghosts P. A. KING,* O. F. FRÖHLICH, and R. B. GUNN, *Department of Physiology, Emory University School of Medicine, Atlanta, Georgia*

The exchange of anions on the band 3 protein requires a conformational change and is inhibited by phloretin; net transport of anions appears to involve tunneling through the protein without a conformational change. We have studied the efflux of $SO_4^{=}$ from gramicidin-treated red blood cell ghosts (containing 120 mM K_2SO_4 and 30 mM HEPES) into high-SO_4 and zero-SO_4 media at low and high V_m (membrane potential) in order to evaluate contributions of tunneling vs. a mechanism requiring conformational change during net flux. The HEPES-buffered (pH 7.0) efflux media were 120 mM K_2SO_4 (low V_m); 80 mM K_3-citrate (low V_m); 120 mM (*N*-methyl-D-glucamine)$_2SO_4$, 4 mM K^+ (high V_m); and 80 NMG_3-citrate, 4 mM K^+ (high V_m). All four conditions were tested in the presence and absence of 200 μM DNDS; only the DNDS-sensitive fluxes are reported. SO_4 effluxes at 20°C into high- and zero-SO_4 were similar at low V_m: 1.5 and 1.8 mmol/(3×10^{13} ghosts·min), respectively. Both values were increased at high V_m to 3.3. Phloretin could either activate or inhibit sulfate efflux. Under zero-SO_4, high-V_m conditions, phloretin (5–300 μM) only *stimulated* efflux ($\leq$10-fold). Under the other three conditions, however, as the phloretin concentration was increased, sulfate efflux was first inhibited and then stimulated. Efflux under high-SO_4, high-V_m conditions recovered to above control (zero phloretin) at high phloretin. The K_i for phloretin inhibition (0–40 μM phloretin) of SO_4 efflux into high SO_4 at low V_m was 9.5 μM. Sucrose substituted for citrate and experiments in CO_2-free media showed that exchange with citrate or residual HCO_3 was insignificant in zero-SO_4 media. We conclude that phloretin inhibits the sulfate net efflux involving conformational changes of band 3, but activates the voltage-sensitive net efflux—i.e., tunneling—through the protein. This is confirmed in experiments where we found that high phloretin concentrations (>100 μM) stimulate Cl^- net efflux from intact cells. [Supported in part by USPHS grants HL28674, GM31269, and HL07342.]

69. Increase in Cytoplasmic Free Ca^{2+} Caused by Halothane in Lymphocytes from Malignant Hyperthermia Patients and Pigs AMIRA KLIP, BEVERLY A. BRITT,* and M. ESTHER ELLIOTT,* *The Hospital for Sick Children, Toronto, and Departments of Biochemistry and Pharmacology, University of Toronto, Toronto, Ontario, Canada*

Malignant hypothermia is a muscle disorder characterized by an abnormal response to anesthetics, stress, and exercise. It is typified by muscle contracture and a dramatic elevation in body temperature. A defect in the regulation of the concentration of cytoplasmic free calcium, $[Ca^{2+}]_i$, is thought to underlie this disease. Using the fluorescent Ca^{2+} indicator quin-2, it is shown here that the anesthetic halothane increases $[Ca^{2+}]_i$ in isolated lymphocytes from malignant hyperthermia-susceptible humans and pigs, but not in their normal counterparts. The halothane-mediated rise in $[Ca^{2+}]_i$ required extracellular Ca^{2+}, and was prevented by nifedipine, an inhibitor of the voltage-sensitive Ca^{2+} channels of the cell membrane. In addition, halothane had an effect on the Ca^{2+} releasable from intracellular stores. This compartment was assayed by the transient increase in $[Ca^{2+}]_i$ caused by the ionophore ionomycin in cells suspended in Ca^{2+}-free, EGTA-containing medium. After the addition of halothane to cells from normal humans and pigs, >70% of the Ca^{2+} of intracellular stores was still available for release by ionomycin. In contrast, only ~45% of the pool was available for release after treating with halothane cells from malignant hyperthermia-susceptible patients or swine. It is concluded that halothane acts both at the cell membrane and at intracellular organelles, and that this action results in a net increase in $[Ca^{2+}]_i$ in malignant hyperthermia, but not in normal, cells. The action at the cell membrane appears to be on the voltage-sensitive Ca^{2+} channels. In addition, the halothane-mediated increase in $[Ca^{2+}]_i$ could be used as the basis for a noninvasive test of malignant hyperthermia. [Supported by the Medical Research Council of Canada and the Muscular Dystrophy Association of Canada.]

70. The Muscarinic Receptor-mediated Increase in Free Cytoplasmic Ca^{2+} Concentration of Rat Heart Is Not Due to Ca^{2+} Release from Sarcoplasmic Reticulum MICHAEL KORTH,* VIRENDRA K. SHARMA,* and SHEY-SHING SHEU, *De-*

partment of Pharmacology, University of Rochester Medical Center, Rochester, New York

Muscarinic receptors of the ventricular myocardium can exhibit three different affinity states for agonists. The highest-affinity and the middle-affinity states represent receptors that couple to and inhibit adenylate cyclase through a process requiring guanine nucleotides, while the low-affinity state seems to be associated with the turnover of the membrane phospholipid phosphatidylinositol. Stimulation of phosphatidylinositol hydrolysis has been proposed to ultimately cause an increase in intracellular Ca^{2+}. In the present study, the ability of carbachol (0.3 mmol/liter) to stimulate Ca^{2+} release from the sarcoplasmic reticulum of intact cardiac muscle cells was tested. For that purpose, the intracellular Ca^{2+} concentration (Ca_i) was monitored in rat ventricular myocytes by means of the fluorescent indicator quin-2. The results show that carbachol caused Ca_i to rise by 20–25% within 7 min. The increase in Ca_i was due to stimulation of muscarinic receptor, since atropine either prevented or abolished this effect. The carbachol-induced rise in Ca_i remained unchanged in the presence of either 10 mmol/liter caffeine (to deplete Ca^{2+} storage in the sarcoplasmic reticulum) or 1 μmol/liter ryanodine (to prevent Ca^{2+} release from the sarcoplasmic reticulum). The findings indicate that the rise in Ca_i was not due to Ca^{2+} release from the sarcoplasmic reticulum but was probably due to an action of carbachol on transsarcolemmal Ca^{2+} transport. [Supported by Deutsche Forschungsgemeinschaft, NIH grant HL-33333, an AHA Established Investigatorship Award, and an AHA/Genesee Valley Chapter grant-in-aid.]

71. Ca^{2+}-dependent Regulation of Epithelial Na^+ Channels

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Processes involved in the regulation of epithelial Na^+ channels were investigated by measuring amiloride-sensitive $^{22}Na^+$ fluxes in toad bladder vesicles and conducting parallel analyses of membrane-associated phosphorylation-dephosphorylation activities. The data on $^{22}Na^+$ fluxes indicate the presence of a Ca^{2+}-dependent mechanism that down-regulates channels. This process occurs in partially permeabilized whole cells, which results in a stable modification of the apical membrane. This change is preserved in isolated vesicles, even when the vesicles are assayed in Ca^{2+}-free solutions. Maximal channel-mediated $^{22}Na^+$ fluxes were obtained only when permeabilized cells were incubated at 25°C in Ca^{2+}-free EGTA medium before homogenization. The inclusion of micromolar concentrations of Ca^{2+} in the cell-incubating medium (followed by removal before the homogenization) blocked, almost completely, this amiloride-sensitive flux in the vesicles. This effect had a maximal K_i of 8×10^{-6} M Ca^{2+} and a Hill coefficient of 3.5–4.0. The involvement of Ca^{2+}-dependent protein kinases in the above-described effect was excluded on the basis of the following results. (*a*) Incubating cells with phorbol ester had no effect on the Ca^{2+} inhibition constant. (*b*) Calmodulin inhibitor had no effect on transport in the presence or absence of Ca^{2+}. (*c*) Trapping purified protein kinase C, ATP, Ca^{2+}, and phorbol ester in the isolated vesicles did not mimic the inhibition induced by incubating whole cells with Ca^{2+}. Preliminary data indicate that Ca^{2+} inhibition of transport acts via a phosphatase-mediated pathway. Investigation of membrane-associated phosphatases indicated the presence of a Ca^{2+}-activated, vanadate-insensitive phosphatase. In addition, we observed Ca^{2+}-dependent dephosphorylation of membranal 51-kD phosphoprotein, which corresponds to the phosphoprotein reported by Lorenzo et al. (1975. *J. Gen. Physiol.* 65:153), which they tentatively identified as the resulatory subunit of cAMP-dependent protein kinase. Preincubation of whole bladders with 8-Br-cAMP was shown to double the channel-mediated fluxes in EGTA-activated vesicles. Conditions of preincubation (Ca^{2+} or EGTA) also affected the potency of membranes to phosphorylate histone fragment 2b, a specific cAMP-dependent protein kinase substrate. [Supported by NIH grant AM36328 and U.S.-Israel BSF grant 84-00066.]

72. Fractal Model of Ion Channel Kinetics

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The value L measured for a property often depends on the scale ϵ such that $L(\epsilon) = A\epsilon^{1-D}$, where D is the fractal dimension (Mandelbrot. 1983. The Fractal Geometry of Nature). For example, this scaling law holds for the perimeters of clouds, the branching of bronchi, and the energy of earthquakes. If ion channels are also fractal, then as we observe the current at higher-frequency resolution, we would see the channel fluctuate between open and closed states at a faster rate. With the assumption that the kinetic rate constant $k(t) = At^{1-D}$, we derive the predicted frequency histogram of open and closed times for a fractal channel, namely $f(t) = At^{1-D}\exp\{-[A/(2-D)]t^{2-D}\}$. We show how to test data to determine whether the channels are best represented by discrete multiple Markov states such as closed ⇄ closed ⇄ open or by the closed ⇄ open fractal model. Our single channel data from the corneal endothelium are best fitted by the fractal model. Thus, it is not necessary to postulate the existence of multiple closed states to fit the closed time histograms. In the Markov model, the transition probability per unit time depends only on the state of the channel. In the fractal model, it also depends on how long the channel has been in that state. It is as if the protein conformation relaxed to the new state, lowering the transition probability out of that state. [Supported by NIH grants EY4624 and EY1080.]

73. Cytosolic Ca^{2+} in Rat Proximal Tubules from Spontaneously Hypertensive Rats (SHR) Measured Using Fura-2

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An increase in cytosolic Ca^{2+} in platelets from hypertensive humans and in SHR, a widely used model of genetic hypertension, has recently been demonstrated. The implication of this finding is that a rise in cytosolic Ca^{2+} has a proximate role in the development of hypertension and is expressed as a generalized cell defect. Ca^{2+} in renal tubular cells from hypertensive rats is unknown. We measured Ca^{2+} in renal cells from SHR at 6 ($n = 8$) and 20 wk ($n = 8$) of age and in normotensive controls (WKY) at 6 ($n = 7$) and 20 wk ($n = 8$) of age to investigate the impact of increasing blood pressure (BP) on cell Ca^{2+} (or vice versa). Renal cell suspensions of proximal tubules were prepared by standard density gradient centrifugation procedures and incubated with the acetoxymethyl ester of fura-2 for 30 min at room temperature. Fura-2 fluorescence was monitored at 510 nM using excitation wavelengths of 340 and 380 nm. The results are shown in the table.

	Age	BP	Ca^{2+}	Age	BP	Ca^{2+}
	wk	*mmHg*	*nM*	*wk*	*mmHg*	*nM*
SHR	6	135±9	122±14	20	175±6*	126±28
WKY	6	96±5‡	146±21	20	126±4*‡	112±10

* $p < 0.01$ (6 wk vs. 20 wk).
‡ $p < 0.01$ (SHR vs. WKY).

By linear regression analysis, we found no significant correlation between BP and cell Ca^{2+} in either SHR ($r = 0.185$) or WKY ($r = 0.366$). Our data indicate that a rise in cytosolic Ca^{2+} is not a generalized cell phenomenon in genetic hypertension, and that in renal tubular cells, unlike platelets, there is no direct correlation between BP and cytosolic Ca^{2+}.

74. Cytoplasmic Calcium Responses of Individual Mouse Fibroblasts to Nucleotides and Serum

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A transient two- to fourfold rise in the cytoplasmic calcium concentration (Ca^{2+}_{in}) was observed after the addition of 5–100 μM ATP or UTP or 0.4% fetal bovine serum to serum-starved 3T6 mouse fibroblasts. Ca^{2+}_{in} was measured in individual cells by monitoring the fluorescence of the

calcium indicator fura-2, using a quantitative video fluorescence microscopy system equipped for digital image-processing and analysis. This system provided accurate recordings of Ca^{2+}_{int} at up to 5-s intervals and used a flow-through perfusion chamber to enable rapid changing of agonist-containing media. The addition of exogenous ATP to 3T6 cells incubated in either Ca^{2+}-free or Ca^{2+}-containing isotonic media elicited a dose-dependent, transient increase in Ca^{2+}_{int}. The cells responded to ATP with an initial sharp increase in Ca^{2+}_{int} that peaked after 15–45 s, followed by a slow decline to resting levels. Subsequent treatment with ATP did not cause further calcium transients, although a secondary increase in Ca^{2+}_{int} did occur after the addition of 0.4% serum. The nucleotides BzATP [3′-*O*-(4-benzoyl)benzoyl ATP], GTP, ITP, CTP, and ADP did not elicit an alteration in the Ca^{2+}_{int} of 3T6 cells. However, cells did become insensitive to ATP after treatment with BzATP and GTP, but not ADP. UTP also desensitized the cells to ATP. In these experiments, the cells were washed 60 s after the initial nucleotide addition and ATP was applied 40 s later. A considerable cell-to-cell variation in the time and extent of the response was noted. [Supported by grants from the Biotechnology Program "Cornell," NIH, NSF, and ONR.]

75. A Minimal Kinetic Model of [Na + K + 2 Cl] Cotransport with Ordered Binding and Glide Symmetry C. LYTLE* and T. J. MCMANUS, *Department of Physiology, Duke University Medical Center, Durham, North Carolina*

A prominent feature of [Na + K + 2 Cl] cotransport in the duck red cell is a concurrent 1:1 obligate cation exchange that comes in two flavors: K/K (or K/Rb) and Na/Na (or Na/Li). In high-K cells, the predominant mode is K/K. In high-Na cells (nystatin technique), the predominant mode is Na/Na. We have proposed that these exchanges represent partial reactions of the [Na + K + 2 Cl] carrier (Lytle et al. 1986. *Fed. Proc.* 45:548). The absolute ion requirements of the two modes of exchange are remarkably different: in the external medium, K/K exchange requires only K and Cl, but all three ions (Na, K, and Cl) must be present in the cell. In contrast, Na/Na exchange requires only Na in the cell, but all three ions must be present externally. The stoichiometry of net cotransport is always 1 Na:1 K:2 Cl. Partially loaded forms are not transportable, since there is no evidence for electrogenic and/or independent net transport of [Na + Cl] or [K + Cl] by the [Na + K + 2 Cl] cotransport system in this cell. These findings can be reconciled by a simple model. (*a*) Fully loaded carriers oscillate between inwardly and outwardly facing conformations faster than empty carriers. (*b*) An inwardly facing empty carrier will bind ions in the following order: first Cl, then K, then another Cl, and finally Na. (*c*) After transport to the outside, the ions will be released in the same order they were bound: Cl, K, Cl, Na. Therefore, the model shows "first on, first off" or glide symmetry. Depending on the prevailing co-ion concentrations, the carrier may unload completely, allowing the empty form to return (thus performing net cotransport), or unload partially, e.g., debind a Cl and K, then reload, e.g., bind an Rb and another Cl, and return to the inside as the fully loaded form (thus performing K/Rb exchange). In this way, the binding order can explain the co-ion dependence of both obligate exchanges. [Supported by NIH grant HL-28391.]

76. Altered In Situ Phospho-Dephosphorylation of Cardiomyocytes by Autonomic and Other Stimuli JANICE E. MACKAY* and PRAKASH V. SULAKHE,* *Department of Physiology, College of Medicine, University of Saskatchewan, Saskatoon, Canada* (Sponsor: A. Fabiato)

Rat heart ventricular myocytes were isolated in order to investigate which proteins undergo modulation in terms of phospho-dephosphorylation evoked by autonomic stimuli, cytoskeleton disruption, altered membrane permeability, and phorbol esters. For this purpose, intact isolated myocytes were first treated with agents that disrupt microtubules (colchicine), increase membrane permeability (saponin), or stimulate kinase C (phorbol esters [PMA]); then they were loaded with [^{32}P]sodium phosphate and exposed to stimuli known to increase cAMP (isoproterenol, forskolin), decrease cAMP (carbachol [Cch]), increase Ca^{2+} influx by slow channel activa-

tion (BAY K8644), decrease Ca^{2+} influx (verapamil, nifedipine), or stimulate phosphoinositide turnover (Cch). The phosphorylation of proteins (electrophoretically resolved followed by autoradiography) of M_r 150 (probably C protein), 85, 45, a doublet 28 (probably TN_I)/26, and a group around 20 (cytosolic) responds to various stimuli. The phosphorylation of M_r 150 and 28 is enhanced by the cAMP stimulants BAY K8644 and PMA; Cch attenuates the effect of cAMP stimulants. M_r 26 undergoes enhanced phosphorylation in the presence of isoproterenol, forskolin, and calcium channel–directed drugs, especially in colchicine-treated myocytes. M_r 30, 24, and 16 undergo dephosphorylation in the presence of cAMP stimulants and show enhanced phosphorylation in PMA-treated cells. Low saponin or colchicine augments overall incorporation of ^{32}P into myocyte proteins. Surprisingly, phospholamban-type phosphorylation represents a minor component. These myocytes show rapid $^{45}CaCl_2$ influx, which is augmented by isoproterenol and low saponin; the latter also allows detection of Ca^{2+} efflux. Verapamil decreases calcium influx without any discernible effect on the efflux. In our current experiments, we are attempting to determine whether calcium channel peptides undergo phospho-dephosphorylation. We will discuss the roles of channel peptides, phospholamban, and TN_I in Ca^{2+} fluxes and actions in cardiomyocytes. [Supported by an MRC grant. J.M. is a recipient of an SHF studentship.]

77. Mechanism of Calcium Potentiation of Oxygen-free Radical Effects on Renal Mitochondrial Membrane Function: a Model for Post-Ischemic and Toxic Mitochondrial Damage CHARLES D. MALIS* and JOSEPH V. BONVENTRE,* *Renal Division, Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts* (Sponsor: A. Leaf)

With a variety of forms of ischemic and toxic tissue injury, cellular accumulation of Ca^{2+} and the generation of oxygen-free radicals (generated by hypoxanthine and xanthine oxidase) may have adverse effects upon cellular and, in particular, mitochondrial membranes. Damage to mitochondria, resulting in impaired ATP synthesis and diminished activity of cellular energy-dependent processes, could contribute to cell death. In order to model, in vitro, conditions present during post-ischemic or toxic injury, the interactions between Ca^{2+} and oxygen-free radicals on isolated renal mitochondria were characterized. With the site I substrates pyruvate and malate, Ca^{2+} pretreatment with an amount of Ca^{2+} that did not of itself alter mitochondrial function, followed by exposure to oxygen-free radicals, resulted in an inhibition of electron transport chain function and complete uncoupling of oxidative phosphorylation. These effects were partially mitigated by dibucaine, a phospholipase A_2 inhibitor. With the site II substrate succinate, the electron transport chain defect was not manifest and respiration remained partially coupled. The electron transport chain defect produced by Ca^{2+} and oxygen-free radicals was localized to NADH CoQ reductase with a reduction in activity to 35% of control levels. Neither Ca^{2+} nor oxygen-free radicals alone affected the enzyme. Calcium and oxygen-free radicals reduced mitochondrial ATPase activity by 55% and adenine nucleotide translocase activity by 65%. By contrast, oxygen-free radicals alone reduced ATPase activity by 32% and had no deleterious effects on translocase activity. Dibucaine partially prevented the Ca^{2+}-dependent reduction in ATPase activity and totally prevented the Ca^{2+}-dependent translocase damage observed in the presence of oxygen-free radicals. The mechanism for Ca^{2+}-induced potentiation of oxygen-free radical injury is via activation of phospholipase A_2. [C.D.M. is supported by Clinical Investigator Award AM-01320, and J.V.B. by NIH grant HL-31513.]

78. Cytoplasmic Anions and Substrate-derived PO_4 Are Simultaneously Transported with Na in "Uncoupled" Na Efflux Mediated by the Red Cell Na/K Pump REINALDO MARIN and JOSEPH F. HOFFMAN, *Department of Physiology, Yale University School of Medicine, New Haven, Connecticut*

Garrahan and Glynn (1967. *J. Physiol. [Lond.]*. 192:159) defined in human red cells an efflux of Na that occurred in the absence of external Na_o and K_o. This "uncoupled" Na efflux is

dependent on ATP and is inhibited by 5 mM Na_o and by ouabain. Higher concentrations of Na_o activate Na efflux, presumably by stimulating ATPase activity (Glynn and Karlish. 1976. *J. Physiol. [Lond.]*. 256:465; Blostein. 1983. *J. Biol. Chem.* 258:7948) and Na/Na exchange. We previously reported (1983. *Curr. Top. Membr. Transp.* 19:693) that, in resealed, DIDS-treated ghosts containing SO_4 as the principle anion, uncoupled Na efflux is an electroneutral process and the 5 mM Na_o-sensitive Na efflux is twice the Na_o-sensitive SO_4 efflux. Ouabain-sensitive Na efflux, but not ouabain-sensitive SO_4 efflux, was found to exceed the Na_o-sensitive efflux, leaving the other component(s) responsible for the electroneutrality unidentified. We now report that this remainder quantity of ouabain-sensitive, 5 mM Na_o-insensitive efflux of Na is accompanied by PO_4 derived from the PO_4 of ATP (measured with [γ-^{32}]ATP), apparently accounting for the electroneutrality of the overall process. Na efflux was stimulated in a ouabain-sensitive manner by concentrations of Na_o higher than 5 mM in ghosts containing 500 μM ATP in the presence but not in the absence of 1,000 μM ADP. Even so, ouabain-sensitive, Na_o-insensitive PO_4 efflux from ATP (and Na efflux) continued to occur at the higher concentrations of Na_o whether or not ADP was present. These results imply that two distinct forms of the Na/K pump evidently operate in the "uncoupled" Na efflux mode that are distinguished by the source of the anion transported (cytoplasmic or substrate) and by the sensitivity to Na_o. [Supported by NIH grants HL-09906 and AM-17433.]

79. Phlorizinyl 5′-Benzylazide (PhzBAz) Causes RBC Swelling and Inhibits Sickle Cell Formation CHRIS MAYNARD* and DONALD F. DIEDRICH,* *Department of Pharmacology, University of Kentucky, Lexington, Kentucky* (Sponsor: Ben Kaminer)

In subdued light, the potential photoaffinity labeling agent PhzBAz is a potent (K_i ~0.5 μM), reversible, strictly competitive inhibitor of 3-*O*-methyl glucose equilibrium exchange (and a noncompetitor of glucose efflux) in the human erythrocyte. Although the compound blocks the initial rates of sugar flux, it also induces a delayed increase in the cell volume of these cells (0.2% hct) in isosmotic medium (PBS, pH 6.2 or 7.4); the dose-dependent hemolysis is preceded by a twofold increase in cell volume (Coulter Counter). Both the rate and extent of hemolysis is dependent upon the cold storage time of cells, temperature, and pH of the incubation buffer. Cell lysis is inhibited by low millimolar levels of sucrose, which presumably remains outside the cell, acts as an osmotic balance, and prevents water influx. Phase and scanning electron microscopy verified that spherocytes are formed before the cell bursts. These osmotic effects occur at only a slightly greater concentration than that at which the drug inhibits glucose transport. Our preliminary observations suggested that PhzBAz, acting as a membrane-perturbant, could be a potentially useful antisickling agent. By allowing less than maximal salt and water entry into the sickle cell, PhzBAz would dilute hemoglobin S and thereby inhibit the highly concentration-dependent aggregation of deoxy-Hgb/S. To determine whether the drug could cause this effect, erythrocytes from sickle cell SS and SC patients were deoxygenated in the presence of low doses (12.5 μM) of the agent. Although cell sickling was inhibited and uniformly sized spherocytes were formed, no lysis of the sickle cells occurred. [Supported by BRSG grant RR05374, NIH.]

80. When Does the Sperm Fuse with the Egg? DAVID H. McCULLOH* and EDWARD L. CHAMBERS, *Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida*

The earliest physiological event during fertilization of the sea urchin egg is a depolarization of the egg's plasma membrane, which is caused by a conductance increase that occurs within 3 s after sperm attachment. It is not clear whether this conductance increase is caused by sperm ligands binding to and "gating" ion channels in the egg's plasma membrane or by the sperm membrane fusing with and adding ion channels to the egg's plasma membrane. The purpose of this study was to distinguish between these two mechanisms in *Lytechinus variegatus* by determining when the membranes of the sperm and egg fuse relative to the conductance increase.

An increase of the egg's membrane capacitance indicated when sperm and egg membranes fused. The egg was voltage-clamped at a potential that permits sperm entry (−10 mV relative to the bath). A small patch of membrane was isolated from the bath by pushing a pipette (5–10 μm tip diam) containing seawater against the egg's surface. The potential inside this pipette was voltage-clamped using a sine wave (1,000 Hz, 20 mV amplitude) centered at the bath potential. A lock-in amplifier continuously monitored the capacitance of the patch by analyzing the sinusoidal current during and after infusing sperm in the pipette. When a sperm caused a step-like inward current (conductance increase) in the egg, current from the pipette initiated simultaneously and had the identical amplitude during the first 5–10 s. A step-like increase of capacitance for the patch simultaneously accompanied the conductance increase. These observations indicate that the initial conductance increase occurs at or near the site of sperm attachment. In addition, the simultaneity of the capacitance and conductance increases suggests that the initial conductance increase occurs when sperm ion channels are incorporated into the egg's plasma membrane during membrane fusion. [Supported by NIH F32-HD06505, NIH R01-HD19126, and NSF DCB-831864.]

81. Na/Ca Exchange and Ca Pump Fluxes in Ferret Red Blood Cells M. A. MILANICK and J. F. HOFFMAN, *Department of Physiology, Yale University School of Medicine, New Haven, Connecticut*

Ferret red cells, like the red cells of dogs, cats, and seals, have $[Na]_i$ = 140 mmol/liter cell water and K_i = 7–8 mmol/liter cell water, and lack a ouabain-sensitive Na/K pump. We sought to determine whether ferret red cells had an Na/Ca exchange system and a Ca pump similar to the transport systems characterized in dog red cells (e.g., Parker. 1979. *Am. J. Physiol.* 237:C10). Cells were loaded with NO_3 replacing Cl at 0°C (to eliminate cotransport), loaded with vanadate at 37°C (to inhibit the Ca pump), treated with DIDS at 37°C (to block vanadate exit), and then washed with $LiNO_3$ at 0°C (to remove Na_o). At 37°C, Ca_o stimulated net Ca efflux approximately twofold in the absence of Na_o. Ca influx was inhibited either by replacing Li_o with Na_o or by replacing Na_i with Li_i (using the reversible sulfhydryl reagent PCMBS). This Na_i-stimulated, Na_o-inhibitable Ca influx appeared to be uphill: after 20 min in 50 μM Ca, cell Ca increased from ~10 to 500 μmol/liter cell water. 100 μM Ca_o was sufficient to half-activate the Ca influx in Na-free media and 2 mM Ca_o was required when Na_o was 150 mM. As there was little increase in the Ca influx when Ca_o was increased from 2 to 100 mM (Na_o = 0), the linear portion of the Ca flux (Ca leak) is small. When Ca_o = 2 mM and Na_o = 150 mM, the Ca influx was 0.3 mmol/(liter cells·h). The coupling stoichiometry varied between 3 and 5 Na/Ca. In the absence of vanadate, no net Ca influx was observed, which supports the notion that these cells contain a Ca pump. Ca was found to stimulate the ATPase activity of hemoglobin-free, porous, broken ferret red cell ghosts. La and vanadate inhibited this stimulation. The coupling ratio of 3–5 Ca/Na implies a net Na efflux of 1–2 mmol/(liter cells·h), which is evidently sufficient to balance the Na leak and, along with the Ca pump, maintain cell volume in the steady state. [Supported by NIH grants HL-09906 and AM-17433.]

82. Quantitative Video Imaging of Free Ionized Calcium in Secreting Tumor Mast Cells PAUL J. MILLARD,* DAVID GROSS,* WATT W. WEBB,* and CLARE FEWTRELL,* *Department of Pharmacology and School of Applied and Engineering Physics, Cornell University, Ithaca, New York* (Sponsor: G. W. G. Sharp)

Changes in the level of intracellular free calcium ($[Ca^{2+}]_i$) associated with the secretion of mediators of immediate hypersensitivity by rat basophilic leukemia (RBL-2H3) cells were measured using the fluorescent indicator fura-2. Digital video imaging allowed quantitative fluorescence ratio maps of $[Ca^{2+}]_i$ in RBL cells to be generated from cell-associated fluorescence imaged in a Zeiss IM35 microscope equipped with a computer-controlled quartz epi-illumination system. Images detected by a low-light-level video camera were digitized and processed to produce quantitative, time-lapse maps of cytoplasmic Ca^{2+} concentrations. Individual cells

responded to a concentration of antigen that induces maximum secretion with a rapid rise in $[Ca^{2+}]_i$, preceded by lag periods ranging from a few seconds to a minute or more. The duration and variability of this lag could be modulated by controlling the rate at which surface IgE receptors were aggregated by antigen. Although extracellular Ca^{2+} is required for prolonged elevation of $[Ca^{2+}]_i$, a component of the calcium signal in antigen-triggered cells appeared to originate from an intracellular store that could be rapidly depleted of calcium when the cells were incubated in Ca^{2+}-free medium. The reduction of $[Ca^{2+}]_i$ to resting levels by treatment with excess monovalent hapten confirmed that continuous aggregation of IgE receptors is essential for maintaining an elevated level of $[Ca^{2+}]_i$ in antigen-triggered cells. [Supported by grants from the Cornell Biotechnology Program, NIH, and ONR. P.J.M. is a PMA Foundation Postdoctoral Fellow.]

83. T Cell Dysfunction in Aged Mice: Altered Production of Intracellular Calcium Transients

RICHARD A. MILLER* and ELIZABETH R. SIMONS,* *Departments of Pathology and Biochemistry, Boston University School of Medicine, Boston, Massachusetts* (Sponsor: Benjamin Kaminer)

Current models for T cell proliferation suggest that protein kinase C (PK-C) and intracytoplasmic Ca^{++} may both play key roles in signal transduction. To learn more about the relative inability of T lymphocytes from old mice to proliferate, we stimulated T cells from old and young mice through the synergistic effects of the PK-C activator phorbol myristate acetate (PMA) and the calcium ionophore ionomycin. Young T cells respond about as well to optimal doses of PMA and ionomycin as they do to the mitogenic lectin Con A, but old T cells respond much better to PMA/ionomycin than to Con A. This suggested that an inability to respond to Con A by activation of PK-C and/or Ca^{++} may underlie age-associated immune dysfunction. Direct tests of intracytoplasmic free Ca^{++} in Con A–stimulated T cells, using indo-1 as a fluorescent indicator, supported this idea: free Ca^{++} is substantially higher in young T cells than in old T cells after Con A exposure. Flow cytometric analyses showed that this defect reflects a diminished number of T cells, in old mice, that are able to elevate their intracellular Ca^{++} concentrations after Con A stimulation. The results show that the first signs of age-associated nonresponsiveness can be detected within minutes of mitogen addition, and suggest that further study of trans-membrane signal transduction may provide a mechanistic insight into age-related immune decline.

84. Characterization of the IgE Receptor-activated Calcium Permeability Pathway in Rat Basophilic Leukemia Cells

F. CHARLES MOHR* and CLARE FEWTRELL,* *Department of Pharmacology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York* (Sponsor: G. W. G. Sharp)

Rat basophilic leukemia (RBL) cells secrete mediators of inflammation in response to antigen-induced aggregation of immunoglobulin E (IgE) receptors. Secretion requires the influx of extracellular calcium through a permeability pathway that has not been fully characterized in these cells. Using the fluorescent potential-sensitive dye bis-oxonol, we have shown that IgE receptor aggregation depolarizes RBL cells. Both depolarization and ^{45}Ca uptake were inhibited by lanthanum or by disrupting the IgE receptor aggregates with monovalent hapten. These results suggest that antigen-stimulated depolarization may be due to the influx of calcium. In agreement with this, we found that RBL cells will still depolarize when all the sodium is replaced with glucose, provided that extracellular calcium is present. In a sodium-containing saline, the cells will depolarize even in the absence of calcium, which suggests that under these conditions Na^+ is able to move through the antigen-stimulated calcium permeability pathway. There appears to be an energy requirement for stimulated calcium influx since depleting the RBL cells of ATP prevented both antigen-induced depolarization and ^{45}Ca uptake. Secretion does not occur in RBL cells that have been depolarized in a high-K^+ solution, and this suggests that these cells do not have voltage-regulated calcium channels. In addition, antigen-stimulated

secretion was inhibited in K^+-depolarized cells. The stimulated uptake of ^{45}Ca correlated closely with the membrane potential and was almost completely inhibited in fully depolarized cells. Increasing the extracellular calcium concentration restored antigen-stimulated secretion and ^{45}Ca uptake. These findings are consistent with the idea that antigen-induced calcium influx in RBL cells is regulated by the electrochemical gradient. [Supported by NIH grants BRSG 08-S7RR05462F-22 and AI 19910.]

85. Na^+-conductive Pathway with Low Affinity for Amiloride in LLC-PK_1 Epithelia ARIE MORAN,* CAROL ASHER, and HAIM GARTY, *Department of Physiology, Beer Sheva Medical School and Department of Membrane Research, The Weizman Institute of Science, Israel*

The diuretic amiloride is known to block different Na^+ pathways, such as Na^+ channels, Na^+/H^+ exchangers, and Na^+/Ca^{++} exchangers. The different sensitivities of these systems to amiloride are often used to distinguish them. In general, Na^+ channels are blocked by <0.1 μM amiloride, while inhibition of Na^+/H^+ and Na^+/Ca^{++} exchangers requires much higher concentrations of the diuretic (10–500 μM). We report here an Na^+-conductive pathway in isolated membranes from LLC-PK_1 epithelia, which is characterized by a relatively low affinity for amiloride, but is different from both the Na^+/Ca^{++} and Na^+/H^+ exchangers. Amiloride-sensitive $^{22}Na^+$ uptake could be demonstrated only in the presence of a negative inside membrane potential. Accordingly, the vesicles were prepared to contain 90 mM of either KCl or NaCl and the flux was measured after substituting all external ions by $Tris^+$ and adding valinomycin (needed only in the K^+-containing vesicles). 60–90% of the initial $^{22}Na^+$ uptake is blocked by 250 μM amiloride. The diuretic dose-response relationships could be fitted to Michaelis-Menten kinetics with a K_i of ~100 μM amiloride. Two lines of evidence indicate that this amiloride-sensitive Na flux is mediated by a conductive pathway: (*a*) a K^+ gradient can drive $^{22}Na^+$ uptake only in the presence of valinomycin; (*b*) the Na^+ gradient–driven $^{22}Na^+$ influx can be blocked by external KCl in the presence of valinomycin. Adding KCl plus valinomycin to the external medium of vesicles that had been loaded with $^{22}Na^+$ induces a fast amiloride-sensitive tracer efflux. The above transport system does not require the presence of Ca or Cl ions. The data are consistent with the possibility that LLC-PK_1 membranes contain an amiloride-sensitive Na^+-conducting pathway different from those reported in the literature. A similar pathway was also observed in membrane vesicles isolated from MDCK cells, rat cortex, rabbit colon, and toad urinary bladder. [Supported by NIH grant AM36328 and U.S.-Israel BSF grant 84-00066.]

86. ATP-dependent Ca^{2+} Sequestration by Endoplasmic Reticulum (ER) Is an Early Event in Renal Hypertrophy D. W. MOSKOWITZ,* S. WESTBROOK,* S. MILLS,* and K. A. KRUSKA,* *Renal Division, Washington University School of Medicine, St. Louis, Missouri* (Sponsor: Philip D. Stahl)

Uninephrectomy uniformly leads to compensatory hypertrophy of the remaining kidney. The signals for compensatory hypertrophy are poorly understood. Since early signals of growth stimuli in other cells involve changes in cytosolic calcium, we reasoned that the growth stimulus to the renal proximal tubule might be reflected in that cell's calcium homeostasis. The ER serves as a Ca^{2+} store for the cell, and it releases Ca^{2+} in response to stimuli associated with growth such as inositol trisphosphate. Thus, an ER fraction enriched 19-fold in cytochrome *c* reductase and de-enriched (0.64-fold) in Na^+/K^+-ATPase was prepared from renal cortical homogenates by sucrose density gradient ultracentrifugation using CsCl, according to the method of Muallem et al. (1985. *Proc. Natl. Acad. Sci. USA.* 82:4433). Electron microscopy revealed vesicles with ribosomes of rough ER. The ER vesicles exhibited ATP-dependent, azide-insensitive uptake of Ca^{2+}, which was promptly released by 10 μM ionomycin and was maximal at 2 min. Mongrel dogs of both sexes underwent uninephrectomy (first kidney), followed either 30 min or 24 h later by removal of the second kidney. In ER from normal dogs, Ca^{2+} uptake

was increased significantly in the second kidney at 30 min but not at 24 h. ER from the second kidney of dogs thyroparathyroidectomized 48 h previously also had increased Ca^{2+} uptake at 30 min after uninephrectomy, which indicates that parathyroid hormone and calcitonin were not required for the early growth signal. ER from PTH-intact dogs, however, had approximately twice the activity of Ca^{2+} uptake as ER from thyroparathyroidectomized dogs. These findings suggest that the chronic presence of PTH may be permissive for growth, although acutely PTH may not represent a growth signal. These findings further suggest that increased sequestration of Ca^{2+} by the ER is an early event in, and is required for the subsequent orchestration of, renal compensatory growth. [Supported by NIH grant AM032087.]

87. Ca^{2+} Reloading of Intracellular Stores During and After Cell Stimulation SHMUEL MUALLEM, CLAUS J. FIMMEL, MARRY SCHOEFFIELD, and STEPHEN PANDOL, *Division of Cell Biology, Cedars Sinai Medical Center, UCLA School of Medicine, Los Angeles, California, and Veterans Administration Medical Center, San Diego, California* (Sponsor: George Sachs)

We previously reported that carbachol stimulation of ponacatic acid resulted in a transient increase in free cytosolic Ca^{2+} ($[Ca^{2+}]_i$), which was due to Ca^{2+} released from the endoplasmic reticulum (ER). The carbachol-stimulated acini failed to respond to a second hormone (CCK-OP) unless they were incubated with atropine. Here, using fura-2–loaded acini, we studied the dependence of the recovery of the CCK-OP effect on the presence of medium Ca^{2+}. When acini suspended in Ca^{2+}-free medium were stimulated with carbachol, a transient increase in $[Ca^{2+}]_i$ was observed. When these acini were inhibited with atropine for 5 min, they failed to respond to CCK-OP, which indicates that the ER was completely depleted of Ca^{2+} during the stimulation period in Ca^{2+}-free medium. The addition of Ca^{2+} to acini treated with carbachol and atropine in Ca^{2+}-free medium resulted in a complete recovery of the CCK-OP signal. This Ca^{2+} uptake from the medium into the cytosol and then into the ER could be blocked by Ca^{2+} channel blockers, which indicates the participation of a plasma membrane Ca^{2+} channel in the reloading process of Ca^{2+}-depleted ER. When acini suspended in medium containing 2 mM Ca^{2+} were stimulated with carbachol for 5 min and then atropine and a sufficient amount of EGTA were added to terminate stimulation and remove all medium Ca^{2+}, the recovery of the CCK-OP was ~60% of control. Further, after carbachol treatment of acini in medium containing 2 mM Ca^{2+}, the addition of 250 nM ionomycin and 2.2 mM EGTA resulted in an increase in $[Ca^{2+}]_i$ that was ~60% of that induced by carbachol or ionomycin addition to resting cells. These experiments indicate that the ER was partially (60%) loaded with Ca^{2+} during the stimulation period and only when Ca^{2+} was present during stimulation. This partial reloading required the activation of plasma membrane Ca^{2+} channel and the ER Ca^{2+} pump.

88. Effect of Anti-Ig on Cytosolic Ca^{2+} in Daudi Lymphoblastoid Cells D. A. NACHSHEN, L. M. PFEFFER,* and I. TAMM,* *Department of Physiology, Cornell University Medical College, and Virology Laboratory, The Rockefeller University, New York* (Sponsor: Olaf Andersen)

We examined the response in the free intracellular calcium concentration (Ca_i) of Daudi cells (human lymphoblastoid cells) to antibodies against surface immunoglobulins (anti-Ig), and the relationship of Ca_i to anti-Ig–induced capping. At 80 μM intracellular quin-2, anti-Ig (10 μg/ml) caused a rapid increase in Ca_i from 100 to 600 nM; the signal returned to baseline within 1 min. At 450 μM intracellular quin-2, Ca_i rose to only 250 μM, and declined gradually to baseline after >7 min. In subsequent experiments, the lower concentrations of quin-2 were employed. A plot of the amplitude of the Ca_i transients (ΔCa_i) and of the binding of anti-Ig to Daudi cells vs. the anti-Ig concentration showed similar saturation kinetics (half-saturation, 2–3 μg/ml). Part of the ΔCa_i is derived from extracellular Ca, and part from nonmitochondrial intracellular stores. Caffeine (4 mM) and TMB-8 (0.5 mM) suppressed the release of Ca from internal stores and the entry of Ca from outside of the cells, but permitted capping in more than half of the

cells. Conversely, interferon-α (250 U/ml) inhibited capping, but did not block ΔCa_i. Phorbol esters (1–2 nM) inhibited both receptor capping and ΔCa_i. None of these agents blocked the binding of anti-Ig to the cells. It appears that receptor capping is not dependent on Ca_i. [Supported by USPHS grants NS 20464, CA 18213, and GM 36716.]

89. Cooperative Action of Calcium Ions in Dopamine Release from Rat Brain Synaptosomes D. NACHSHEN and S. SANCHEZ-ARMASS,* *Physiology Department, Cornell University Medical College, New York*

The release of [^{3}H]dopamine from isolated presynaptic nerve terminals (synaptosomes) prepared from rat striata was measured as a function of the external Ca^{2+} concentration (Ca_o). In synaptosomes that were depolarized by the addition of 50 mM K^+, release increased in a highly nonlinear manner with Ca_o; release could be described as a third-power function of Ca_o. Both the ^{45}Ca influx and the change in the free intraterminal Ca concentration (Ca_i, measured with the fluorescent Ca indicator fura-2) that were evoked by depolarization increased linearly and saturated with increasing Ca_o. These results suggest that the nonlinearity in the Ca_o dependence of neurotransmitter release originates in a cooperative relation between Ca_i and exocytosis. [Supported by NIH grant NS20464 and by an Investigatorship from the American Heart Association, New York Chapter.]

90. Activation and Inactivation of Dihydropyridine-sensitive Single Calcium Channels in Cells Isolated from the Rabbit Mesenteric Artery MARK T. NELSON and JENNINGS F. WORLEY,* *Department of Pharmacology, University of Vermont College of Medicine, Burlington, Vermont*

Single vascular smooth muscle cells were enzymatically isolated from rabbit mesenteric artery. The patch-clamp and bilayer techniques were used to examine currents through single calcium channels. Activation of the 8- and 15-pS channels was voltage dependent, with membrane depolarization accelerating the activation rate (i.e., decreasing the time to first opening). The probability of the 8-pS channel being open (P) increased from ~0.02 at −35 mV to 0.33 at 0 mV. However, P did not reach 1.0, even with depolarizations to +25 mV. The rate of inactivation of the 8-pS channel increased with membrane depolarization. The "calcium antagonist" nisoldipine decreased P, slowed the rate of activation, and increased the frequency of test pulses without openings in a dose-dependent manner. Almost complete inhibition of both types of channels was achieved with 50 nM nisoldipine (holding potential, −75 mV; test potential, 0 mV). The degree of inhibition of 8-pS calcium channel activity was voltage dependent, with 30 nM nisoldipine reducing the mean P by ~70% at 0 mV and by ~28% at −25 mV (holding potential, −75 mV). These results are consistent with these calcium channels being the pathways for calcium influx in vascular smooth muscle and being the targets of the clinically important "calcium antagonists." [Supported by a grant-in-aid (84-879) from the American Heart Association (AHA). M.T.N. is an Established Investigator of the AHA. J.F.W. is a Fellow of the AHA, Florida Affiliate.]

91. Cation Receptor-dependent Changes in Cytosolic Ca^{2+} and the Regulation of Hormone Secretion in Parathyroid Cells E. F. NEMETH* and ANTONIO SCARPA, *Case Western Reserve University School of Medicine, Department of Physiology and Biophysics, Cleveland, Ohio*

Changes in the concentration of extracellular Ca^{2+} regulate the secretion of parathyroid hormone (PTH), although how the extracellular Ca^{2+} signal is detected by the parathyroid cell is unknown. Nor is it clear how this recognition event is subsequently transformed into an intracellular signal(s) that modulates secretion. Our recent studies using the fluorescent indicator fura-2 to measure the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) provide some insight into the

mechanisms underlying these processes. In dissociated bovine parathyroid cells loaded with fura-2, small increases in the concentration of extracellular Ca^{2+} evoke rapid and transient increases that are followed by lower yet sustained increases in $[Ca^{2+}]_i$. Sustained steady state increases in $[Ca^{2+}]_i$ are dependent on the presence of extracellular Ca^{2+} and probably involve Ca^{2+} influx, whereas cytosolic Ca^{2+} transients arise from the mobilization of cellular Ca^{2+}. A variety of other divalent and trivalent cations also evoke transient increases that are not, however, followed by sustained increases in $[Ca^{2+}]_i$. Cation-induced cytosolic Ca^{2+} transients are not seen in quin-2–loaded cells. Transient, but not sustained, increases in $[Ca^{2+}]_i$ are associated with an inhibition of PTH secretion. PTH secretion is still inhibited, however, when cytosolic Ca^{2+} transients are blocked by loading cells with high concentrations of Ca^{2+} chelators. Conversely, cytosolic Ca^{2+} transients elicited by ionomycin (which bypasses receptor-dependent events) are not sufficient to inhibit secretion. The data provide some reason to suppose that there is a receptor on the surface of parathyroid cells that is promiscuous and responds to a variety of divalent and trivalent cations. The activation of this receptor leads to the rapid mobilization of Ca^{2+} from some cellular store, resulting in a transient increase in $[Ca^{2+}]_i$. The degree of activation of this receptor, rather than transient or steady state changes in $[Ca^{2+}]_i$, appears to be the pivotal factor regulating secretion of PTH. [Supported by NIH grant AM-33928.]

92. The Role of Mitochondria in Calcium Homeostasis in Cultured Heart Cells ANNABELLE A. OKADA, DONGHEE KIM, and THOMAS W. SMITH, *Brigham and Women's Hospital, and Harvard Medical School, Boston, Massachusetts*

Mitochondria are capable of electrogenic uptake of calcium in myocardial cells, but their role in sequestering calcium under conditions associated with calcium loading is not well defined. To examine further the role of mitochondria in calcium handling in the intact cell, we measured the effect of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a potent inhibitor of oxidative phosphorylation, on trans-sarcolemmal ^{45}Ca fluxes, free intracellular calcium ($[Ca]_i$), and the amplitude of contraction in cultured chick embryo ventricular cells. After 5 min exposure to 2 μM CCCP, causing a 45% decrease in amplitude of contraction with no change in beating rate, rates of ^{45}Ca uptake and efflux decreased by 42 and 32%, respectively ($p < 0.01$), with a corresponding 31% reduction in the size of the rapidly exchangeable calcium pool measured at 5 min ($p < 0.05$). After 2 h of exposure to 2 μM CCCP, there was no further change in the ^{45}Ca content, which suggests that mitochondria principally contribute to the modulation of rapidly exchangeable Ca (equilibration by 5 min), rather than the slow phase of Ca exchange (equilibration by 2 h) in these heart cells. 2 μM CCCP, a concentration that substantially inhibits oxidative phosphorylation, had no measureable effect on the mean $[Ca]_i$ as measured with fura-2 (SPEX spectrofluorometer), which is consistent with the maintenance of SR Ca sequestration and sarcolemmal Ca extrusion mechanisms by glycolytically generated ATP. Ca loading was induced by Na pump inhibition with 0.1 mM ouabain, followed by exposure to choline-substituted, Na-free medium, which stimulated Ca influx via Na/Ca exchange. Under these conditions, measurements with fura-2 demonstrated that 2 μM CCCP markedly reduced the rate of decrease in $[Ca]_i$ after zero-Na_o exposure. This was accompanied by augmented and sustained contracture signals compared with control cells in the absence of CCCP. We conclude that mitochondria contribute to the maintenance of the rapidly exchangeable (but not slowly exchangeable) Ca pool in cultured heart cells. Under conditions of Ca loading, mitochondria appear to play an important role in buffering within the initial 30 s of Ca loading. [Supported by NIH grant HL18003.]

93. Relationship Between Inositol Phosphate Release and Na/H Exchange in NIH-3T3 Cells Expressing the EJ Human Bladder Ras Oncogene NANCY E. OWEN, JOANNE N. KNAPIK,* and ROBERT R. GORMAN,* *Chicago Medical School, North Chicago, Illinois, and The Upjohn Company, Kalamazoo, Michigan*

Previous studies have suggested that although a Ca-mediated event is a necessary step for serum-stimulated Na/H exchange in Swiss 3T3 cells, this Ca-mediated event is bypassed or its

necessity is greatly reduced in virally transformed SV40-3T3 cells (Owen and Villereal. 1985. *Am. J. Physiol.* 248:C288). Since one means of increasing intracellular Ca is via IP_3-mediated Ca mobilization, in the present study we compared the relationship between IP release and Na/H exchange (benzamil-sensitive net Na influx) in NIH-3T3 cells, in NIH-3T3 cells expressing the normal human Harvey-ras gene (EC cells), and in NIH-3T3 cells expressing the EJ human bladder ras oncogene (EJ cells). Our results are shown below:

	IP release (cpm [^{3}H]inositol/mg prot)			Net Na influx (μmol/g prot/min)		
	Basal	+ Serum	Stimulation	Basal	+ Serum	Stimulation
3T3	750	1,650	220% (n = 10)	7.8	22.6	320% (n = 4)
EC	715	997	140% (n = 7)	7.3	19.2	270% (n = 7)
EJ	1,500	1,650	110% (n = 9)	14.2	70.4	600% (n = 6)

These results demonstrate that (*a*) basal IP release and net Na influx are elevated in EJ cells relative to EC or to 3T3 control cells, (*b*) serum does not stimulate IP release in EJ cells, and (*c*) the extent of serum-stimulated net Na influx is higher in EJ cells than in EC or in 3T3 control cells. These data support the contention that although IP release may be important in serum-stimulated net Na influx in 3T3 or EC cells, it does not appear to be a step in the signal-transduction pathway for Na/H exchange in EJ cells. [Supported by the Chicago Heart Association, the Schweppe Foundation, and NIH grant HL31959.]

94. Single Channel Characterization of a Ca-activated K Channel Involved in Cell Swelling at the Basolateral Membrane of Rabbit Proximal Convoluted Tubule

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(Sponsor: Douglas C. Eaton)

The patch-clamp method was used to investigate the main ionic channel involved in cell swelling at the basolateral membrane of the rabbit proximal convoluted tubule (PCT). The PCTs, microdissected from rabbit kidney, were incubated 20 min in a physiological saline solution (Earles-HEPES) containing Cooper collagenase (456 U/ml). To induce cell swelling, each PCT was then transferred in a high-K Earle-HEPES solution. Under this condition, current jumps associated with an inward rectifier channel could be observed in >80% of the patches in the cell-attached configuration. Single channel *I-V* curves were measured with pipette solutions containing NaCl and KCl in ratios chosen to keep the ionic strength constant (150 mM). The measured inward currents' single channel conductance ranged from 35 to 60 pS with 75 and 200 mM KCl pipette solution, respectively. When the pipette solution was changed from 200 to 5 mM KCl, the zero-current potential shifted from 7 to −50 mV, which indicates that the channel is mainly permeable to K ions. These results could be fitted using the Goldman-Hodgkin-Katz equation with a ratio $P_{Na}/P_K = 0.1$. With a normal Earles-HEPES solution as the bathing medium, the open channel probability decreased to <10%, which indicates that cell swelling may activate this K channel. We also obtained evidence from inside-out patch experiments that internal Ca can trigger K channel activity within the micromolar range. However, the suggestion that an increase in cytoplasmic Ca is the mechanism responsible for the K channel activation in cell swelling remains to be proven. [Supported by an MRC studentship grant.]

95. Ca-Na and Na-H Exchange in Dog Red Blood Cells (RBC): Fixation of the Activating Mechanisms with Sulfhydryl Cross-linkers

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Swelling activates Ca-Na exchange in dog RBC, while shrinkage activates Na-H exchange. Both pathways turn off when the cell volume is returned to normal. Treatment of the cells with thiol-active reagents can result in permanent activation ("fixation") of each of these transporters, so that the ion translocations they mediate are no longer reversible with removal of the volume

stimulus. The swelling-induced Ca-Na exchanger can be locked in the activated or "on" position by treating swollen cells with 0.3 mM diamide, a thiol oxidizer, for 30 min at 37°C. Optimal fixation occurs when diamide is applied to cells suspended in a hypotonic LiCl medium containing 0.5 mM Ca. The action of diamide on Ca-Na exchange is opposed by external Na and by Ca concentrations of <0.2 and >1.0 mM. The effect of diamide can be reversed with dithiothreitol. The shrinkage-induced Na-H exchanger can be locked in the "on" position by treating shrunken cells with bifunctional maleimides, the best of which is *N,N'-o*-phenylene dimaleimide (o-PDM) at 20–22°C for 5 min. Fixation of the Na-H transporter in the "on" configuration shows a sharp optimum at 2–3 μmol o-PDM per ml RBC, occurs best in a hypertonic NaCl medium, and has no Ca requirement. Pretreatment of cells with the monofunctional analogue phenylmaleimide prevents subsequent fixation of the Na-H exchanger by o-PDM. The monofunctional agent alone can lock the Na-H exchanger in the "off" configuration: swollen cells are 5–10 times more sensitive to this effect than are shrunken cells. The results suggest that the Ca-Na and the Na-H exchangers are triggered by activation mechanisms that sense cell volume. As cells are caused to swell and shrink, adjacent sulfhydryl groups must move in a way that determines whether or not cross-linking will take place under the special conditions described.

96. Specialized Subcellular Sites of Calcium Release by Inositol 1,4,5 Trisphosphate and Light in *Limulus* Ventral Photoreceptors RICHARD PAYNE* and ALAN FEIN,* *Marine Biological Laboratory, Woods Hole, Massachusetts* (Sponsor: L. F. Jaffe)

The intracellular messenger inositol 1,4,5 trisphosphate ($InsP_3$) releases calcium from nonmitochondrial stores. We investigated the distribution and identity of $InsP_3$- and light-sensitive calcium stores in *Limulus* ventral photoreceptor cells, where light and $InsP_3$ raise intracellular calcium. We injected ventral photoreceptor cells with the photoprotein aequorin and viewed its luminescence with an image-intensifier (Zeiss TV3M). After 10-ms flashes that illuminated the entire photoreceptor and saturated the receptor potential, aequorin luminescence was confined to the light-sensitive rhabdomeral (R) lobe of the cell, where 10-μm-diam test spots of light were subsequently best able to depolarize the receptor. Typically, luminescence was confined to one R-lobe, an area having a diameter of 40–80 μm within the 80 × 150-μm cross-section of the cell. Injection of 100 μM $InsP_3$ into the photoreceptor only elicited detectable aequorin luminescence when the injection was made into the light-sensitive R-lobe. Injection of $InsP_3$ into the arhabdomeral lobe elicited no detectable luminescence. Calcium stores released by both light and $InsP_3$ are therefore localized to the R-lobe. Cisternae of smooth endoplasmic reticulum beneath the plasma membrane of the R-lobe are a probable candidate for these stores. In addition to the differences between lobes, aequorin luminescence elicited by injection of $InsP_3$ was further confined to a subregion of the R-lobe, within a radius of 20 μm around the injection site, due to rapid dilution and/or metabolism of injected $InsP_3$. Aequorin luminescence elicited from 20-μm-diam light spots centered on the R-lobe was also confined to the area illuminated. [We thank Dr. S. Inoue for the loan of the TV3M, and Dr. O. Shimomura and Dr. C. Ballou for gifts of aequorin and $InsP_3$, respectively. Supported by NIH grant EY03793.]

97. Changes in $[Ca^{2+}]_i$ During Spontaneous Contraction and Zero-$[Na^+]_o$–induced Contracture in Cultured Ventricular Cells Detected with Indo-1 GEORGE A. PEETERS* and WILLIAM H. BARRY, *Cardiology Division, University of Utah, Salt Lake City, Utah*

New fluorescent $[Ca^{2+}]$-sensitive dyes may simplify the measurement of $[Ca^{2+}]_i$ transients in myocardial cells. We exposed spontaneously contracting cultured chick embryo ventricular cells to indo-1 AM (10 μM) for 15 min. This produced a decrease in contractile amplitude to 35 ± 10% control (mean ± SEM). After 30 min wash, contractile amplitude recovered to 53 ± 8% control. Using an inverted 40× objective epifluorescence system, washed cells containing Ca^{2+}-sensitive indo-1 were excited at 360 nm, and fluorescence (F) was measured at 405 nm (↑ with

↑ Ca^{2+}) and 480 nm (↓ with ↑ Ca^{2+}). Calibration of the $[Ca^{2+}]_i$ signals was achieved by the use of Ca^{2+}-buffered solutions containing the nonfluorescent Ca^{2+} ionophore bromo A23187. Comparison of the *F* ratio of 405:480 nm at zero Ca^{2+} (R_{min}) and 1 mM Ca^{2+} (R_{max}) gave different values in vivo (0.17 and 0.78) and in vitro (0.08 and 1.50), which suggests the presence of intracellular noncytosolic dye. Correction for this intracellular compartment was made by recalculating the background *F* at 405 and 480 nm based on a formula derived from the in vitro and in vivo calibration *F* levels. Calculated diastolic and systolic $[Ca^{2+}]_i$ were 355 ± 51 and 700 ± 126 nM (mean ± SEM, $n = 8$). $[Ca^{2+}]_i$ during zero-$[Na^+]_o$–induced contracture was 1,885 ± 426 nm. The onset of the $[Ca^{2+}]_i$ transient preceded motion by 27 ± 5 ms. These results indicate that indo-1 can be used in these cultured cells to measure changes in $[Ca^{2+}]_i$ associated with contraction and contracture. [Supported in part by USPHS grants HL30478 and HL07576.]

98. Augmentation of the Abundance of Na,K-ATPase α-Subunit mRNA by Low External K^+ in a Rat Liver Cell Line THOMAS A. PRESSLEY,* FARAMARZ ISMAIL-BEIGI, GREGORY G. GICK,* and ISIDORE S. EDELMAN, *Departments of Biochemistry and Molecular Biophysics and of Medicine, College of Physicians and Surgeons, Columbia University, New York*

Exposure of the rat liver cell line ARL 15 to external K^+ concentrations below 1 mM for 24 h results in a 60% increase in Na,K-ATPase activity and Na,K pumping capacity (Pressley et al. 1986. *J. Gen. Physiol.* 87:591). That this represents an increase in the number of pump units is suggested by increases in specific ouabain-binding sites in HeLa cells and chick ventricular cells under similar low-K^+ conditions (Pollack et al. 1981. *J. Cell Physiol.* 106:86; Kim et al. 1984. *Circ. Res.* 55:39). Use of a cDNA probe (1,200 nucleotide pairs) complementary to the rat brain mRNA encoding the α-subunit of the Na,K-ATPase ($mRNA_\alpha$) in Northern blot analysis revealed a single 26–27 S band in total RNA isolated from ARL 15 cells exposed to low-K^+ (0.6 mM) or control media (5.4 mM) for 24 h. The abundance of $mRNA_\alpha$ in control and low-K^+–treated cells was estimated by dot blot analysis of total RNA. Exposure of cells to 0.6 mM external K^+ for 24 h augmented the specific $mRNA_\alpha$ content by 60% relative to exposure to control concentrations of K^+. The increase in specific $mRNA_\alpha$ abundance was detectable as early as 6 h after exposure to low external K^+. Low-K^+ exposure also caused a 40% increase in the RNA/DNA ratio. Thus, when expressed per unit DNA, low-K^+ treatment increased the abundance of $mRNA_\alpha$ by ~120%. These data suggest that exposure of cells to low external concentrations of K^+ produces an increase in the abundance of $mRNA_\alpha$ that in turn may mediate a significant part of the increase in the abundance of the Na,K pump. [Supported by NIH grants CA-22376, AM-31089, and GN-36618.]

99. Na-independent pH_i Recovery in Frog Skeletal Muscle ROBERT W. PUTNAM and ALBERT ROOS, *Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri, and Department of Physiology and Biophysics, Wright State University School of Medicine, Dayton, Ohio*

The intracellular pH (pH_i) of frog semitendinosus muscle fibers was measured with glass microelectrodes. The pH_i recovers from 5% CO_2-induced acidification at a rate of ~0.3 pH_i/h (equivalent to an acid efflux of ~10 $pmol \cdot cm^{-2} \cdot s^{-1}$) if the fibers are depolarized to approximately −20 mV in 50 mM K/constant Cl. This recovery is due to two Na-dependent membrane transport systems: Na/H exchange and (Na + HCO_3)/Cl exchange. Recovery from acidification after an NH_4Cl pulse in the nominal absence of CO_2 (average rate, ~0.3 pH_i/h, equivalent to an acid efflux of ~5 $pmol \cdot cm^{-2} \cdot s^{-1}$) is largely due to Na/H exchange. In the absence of Na (substituted by *N*-methyl-D-glucammonium), recovery is greatly slowed (Putnam et al. 1986. *J. Physiol. [Lond.]*. In press). We have recently studied, in frog muscle, the effect on pH_i of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). In fibers superfused with HEPES-buffered medium (pH 7.35, no CO_2, 50 mM K/constant Cl, 70 mM Na), 1 mM IBMX

caused a small transient intracellular alkalinization followed by a slow (~1 h) acidification to a new steady state. The alkalinization can be ascribed to the entry of the weak base IBMX; the slow acidification may be due to metabolic acid production, stimulated by the observed two- to threefold increase in intracellular cAMP induced by IBMX. IBMX increased the rate of recovery two- to threefold after an NH_4Cl pulse. Na removal reduced the recovery rate by ~0.25 pH_i/h both in the absence and presence of IBMX. Thus, in the absence of external Na, a striking pH_i recovery of ~0.5 pH_i/h is seen with IBMX. This implies a previously unsuspected, Na-independent recovery mechanism in frog muscle that can be stimulated by IBMX. The basis of this IBMX effect and the nature of the Na-independent recovery are currently being studied. [Supported by NIH grant HL-00082.]

100. Cytoplasmic Ca^{2+} Is Not an Obligatory Messenger of Insulin Stimulation of Hexose Uptake in 3T3-L1 Adipocytes TOOLSIE RAMLAL* and AMIRA KLIP, *Division of Neurology, The Hospital for Sick Children, Toronto, Ontario, Canada*

Glucose uptake is stimulated by insulin in differentiated 3T3-L1 adipocytes, but not in the precursor, undifferentiated pre-adipose cells. Stimulation of uptake occurs within 30 min of addition of the hormone, and 1×10^{-8} M insulin produces maximal stimulation. Suspension of the cells by trypsinization or scraping did not impair the response to the hormone. Suspended 3T3-L1 cells were loaded with the fluorescent Ca^{2+} indicators quin-2 or indo-1, and the cytoplasmic concentration of free Ca^{2+}, $[Ca^{2+}]_i$, was determined by calibration with ionomycin or digitonin. Indo-1, with a higher fluorescence yield, produces a Ca^{2+}-dependent fluorescence signal without significant buffering of cytoplasmic Ca^{2+}. The resting $[Ca^{2+}]_i$ was 141 ± 5 nM in undifferentiated cells, 119 ± 5 nM in cells in differentiating medium, and 155 ± 10 nM in differentiated adipocytes. Insulin added to quin-2– or indo-1–loaded 3T3-L1 suspended adipocytes had no detectable effect on $[Ca^{2+}]_i$ for at least 10 min. Incubation of 3T3-L1 adipocyte monolayers with insulin in the presence of EGTA in Ca^{2+}-free medium did not impair the stimulation of hexose uptake by the hormone. This indicates that extracellular Ca^{2+} does not play a role in the insulin response. Incubation of cells with quin-2 in EGTA/Ca^{2+}-free medium during exposure to insulin did not prevent stimulation of hexose uptake. Under these conditions, it is calculated that intracellular quin-2 chelates Ca^{2+} and depletes intracellular Ca^{2+} stores, effectively lowering $[Ca^{2+}]_i$. Hence, it is concluded that insulin does not produce changes in $[Ca^{2+}]_i$, and that chelating intracellular Ca^{2+} does not prevent stimulation of hexose uptake by insulin. These results make it unlikely that cytoplasmic Ca^{2+} changes are a signaling event in the transduction of insulin action on glucose uptake in 3T3-L1 adipocytes. [Supported by the Muscular Dystrophy Association of Canada.]

101. Na/Ca Exchange in Barnacle Muscle Cells Has a Stoichiometry of 3 Na^+:1 Ca^{2+} HECTOR RASGADO-FLORES, ELIGIO M. SANTIAGO, and MORDECAI P. BLAUSTEIN, *Departments of Physiology and Medicine and Hypertension Center, University of Maryland Medical School, Baltimore, Maryland*

The Na/Ca exchange system present in the plasmalemma of numerous types of cells is able to transport Ca^{2+} in either direction across the plasmalemma in exchange for Na. The coupled Ca efflux and Na influx mediated by this system are operationally defined as the "forward mode" of exchange; the coupled Ca influx and Na efflux have been termed the "reverse mode." One of the most important factors that determines the direction of (net) Ca transport is the stoichiometry of the exchange. A direct measurement of this stoichiometry has not yet been made, and its value is controversial. We report here that (nontransported) intracellular Ca^{2+} promotes both external Ca-dependent ^{22}Na efflux and internal Na-dependent ^{45}Ca influx in internally perfused barnacle muscle cells. This indicates that the two fluxes are coupled and mediated by the "reversed mode" Na/Ca exchange. Under our experimental conditions ($[Ca^{2+}]_i$ = 1.0 μM; $[Na^+]_i$ = 46 mM in ATP-fueled cells), the ratio of the rates of the Ca_o-dependent Na efflux (54.0 ± 4.5 pmol/cm^2·s, n = 4) to the Na_i-dependent Ca influx (17.0 ±

0.9 pmol/cm^2·s, n = 4) was 3.2:1. This directly demonstrates that the Na/Ca exchange stoichiometry is about 3 Na^+:1 Ca^{2+} in the barnacle muscle. The exchanger may mediate (net) Ca entry during depolarization (in parallel with the voltage-gated Ca channels) and Ca exit at rest and during repolarization (in parallel with the ATP-driven Ca pump). [Supported by NIH grant AM-32276, by the MDA, and by a fellowship to H.R.-F. from the AHA, Maryland Affiliate.]

102. Exogenous Diacylglycerol Elicits Release of Ca^{++} from Intracellular Stores in HL60 Cells DIEGO RESTREPO* and PHILIP A. KNAUF, *Department of Radiation Biology and Biophysics, University of Rochester Medical Center, Rochester, New York*

Phosphoinositide (PI) turnover has been shown to play an important role in inducing transient changes in cytosolic free calcium ([Ca_i]) through inositol triphosphate- (IP_3) induced release of Ca^{++} from the endoplasmic reticulum. We find that diacylglycerol (DG), the other product of hydrolysis of PI, elicits an increase in [Ca_i] in the promyelocytic HL60 cell. Measurements with the fluorescent indicator fura-2 indicate that exogenous L-1-α-1,2-dioctanoyl glycerol (DiC8) elicits an increase in [Ca_i] that follows Michaelis-Menten kinetics as a function of the DiC8 concentration (resting [Ca^{++}] = 85.1 ± 9.8 nM, maximum change in [Ca^{++}] = 98.0 ± 30 nM, K_m = 37.0 ± 7.6 μM, average ± SD, n = 3). 1-Oleoy-2-acetoylglycerol also elicits an increase in [Ca_i]. In contrast, phorbol myristate acetate (100 nM) does not elicit any changes in cytosolic Ca^{++}. DiC8 elicits an increase in [Ca^{++}] even when extracellular Ca^{++} is buffered with EGTA to 8–20 nM. Studies with ^{45}Ca indicate that calcium uptake is lower and that calcium efflux is higher in the presence of DiC8 compared with control. These observations indicate that Ca^{++} is being released from intracellular stores. Studies with saponin-permeabilized HL60 cells indicate that the addition of DiC8 (40 μM) or IP_3 (12.5 μM) elicits Ca^{++} release from a compartment where Ca^{++} is accumulated against chemical equilibrium when ATP is added to the outside media. [Supported by NIH grants 1 F32 CA07924-011, AM 27495, and HL 18208.]

103. Diphenylamine-2-Carboxylate (DPC) Inhibits Apical Membrane Cl^-/HCO_3^- Exchange in *Necturus* Gallbladder Epithelium LUIS REUSS and JAMES L. COSTANTIN,* *Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri*

Double-barreled Cl^--sensitive and pH-sensitive microelectrodes were employed to assess the effects of DPC on apical membrane Cl^- transport in *Necturus* gallbladder epithelium. DPC blocks Cl^- channels in other epithelia (DiStefano et al. 1985. *Pflügers Arch.* 405(Suppl 1):S95). Tissues were superfused with identical solutions on both sides (buffer: 10 mM HCO_3^-/1% CO_2). Mucosal addition of 0.1 mM DPC caused a reversible hyperpolarization of both cell membranes (from −68 ± 4 to −80 ± 3 mV, 3 min after DPC); aCl_i fell from 21 ± 4 to 15 ± 2 mM and pH_i rose from 7.30 ± 0.05 to 7.44 ± 0.05. In the absence of DPC, reducing mucosal solution [Cl^-] from 98 to 10 mM caused hyperpolarization of both cell membranes (8 mV in 3 min), a fall in aCl_i (by 10 mM; initial rate, 10.3 mM/min), and cell alkalinization (ΔpH_i 0.20; initial rate, 0.30 pH units/min). In 0.1 mM DPC, 10 mM Cl^- caused a 3-mV hyperpolarization, a 2-mM fall in aCl_i (initial rate, 3.4 mM/min), and slight cell alkalinization (ΔpH_i 0.02; initial rate, 0.06 pH units/min). In the absence of DPC, reducing mucosal solution [HCO_3^-] from 10 to 1 mM (cyclamate substitution, constant P_{CO_2}) caused a 3-mV depolarization, a 6-mM increase in aCl_i, and cell acidification (ΔpH_i 0.12). In DPC, 1 mM HCO_3^- caused a 3-mV hyperpolarization, a smaller increase in aCl_i (2 mM), and less acidification (ΔpH_i 0.02). In tissues pretreated with theophylline (to elevate intracellular cAMP levels and induce an apical membrane Cl^- conductance), DPC had no effects on the changes in membrane voltage and conductance elicited by changing mucosal [Cl^-]. We conclude that in this epithelium, DPC reversibly inhibits apical membrane Cl^-/HCO_3^- exchange and has no effects on the cAMP-induced Cl^- channel. [Supported by NIH grant AM19580.]

104. Nifedipine and the Voltage Sensor of Skeletal Muscle Excitation-Contraction (E-C) Coupling EDUARDO RIOS and GUSTAVO BRUM,* *Department of Physiology, Rush University, Chicago, Illinois*

The release of calcium from the sarcoplasmic reticulum (SR) to activate muscle contraction is preceded by changes in a voltage sensor located in the membrane of the transverse tubules, resulting in "intramembrane charge movement." We have detected effects of a Ca antagonist dihydropyridine on Ca release from the SR and charge movement. Intramembrane charge movements and Ca release from the SR (Melzer et al. 1984. *Biophys. J.* 45:637) were measured in cut frog skeletal fibers and voltage-clamped in a double vaseline gap, using a Ca-sensitive dye. 10 nM to 10 μM nifedipine, added externally, caused (*a*) a decrease in peak Ca release in fibers at −70 mV holding potential (HP); (*b*) no change in peak release in fibers at HP = −100 mV; (*c*) a decrease in the intramembrane charge that moves between −70 and 0 mV; (*d*) an increase in the charge that moves negative to −90 mV. Changes *a* and *c* were very well correlated. The parallel effects of nifedipine on Ca release and charge movement and the dependence of the effects on HP are consistent with a dihydropyridine receptor being part of the voltage sensor of E-C coupling. [Supported by NIH grant AM32808.]

105. Variations in Calcium Transients in Different Regions of Neurons from the Crab Stomatogastric Ganglion WILLIAM N. ROSS* and KATHERINE GRAUBARD,* *Department of Physiology, New York Medical College, Valhalla, New York, and Department of Zoology, University of Washington, Seattle, Washington* (Sponsor: L. B. Cohen)

Absorbance changes of the metallochromic indicator arsenazo III were used in conjunction with an array of 100 photodiodes to measure changes in intracellular calcium concentration at many positions simultaneously in identified neurons of the crab stomatogastric ganglion. When stimulated intrasomatically, all the cells that we examined, including LP, PY, GM, PD, and VD, showed voltage-dependent absorbance changes at 660 nm over the neuropil region and soma. When calcium was removed from the bath or when 1 mM cadmium was added, these signals disappeared. Together, these experiments showed that there was voltage-dependent calcium entry (presumably through calcium channels) over most of the surface of these cells. When the membrane potential was allowed to oscillate without stimulation, absorbance oscillations were detected all over the neuropil but not in the soma (Graubard and Ross. *Proc. Natl. Acad. Sci. USA.* 82:5565). In looking for an explanation for this result, we measured the threshold for calcium entry into cells by grading the amplitude of 300-ms current pulses applied to the soma of identified neurons. Interestingly, in a number of cells, we found that the current threshold for detecting a change in absorbance was lower for neuropil regions than for the soma, even though the somatic depolarization would be expected to be attenuated in spreading to the neuropil. This result persisted in TTX, which shows that it was not higher action potential amplitudes in the neuropil that caused the lower threshold there. This suggests that the voltage threshold for calcium entry may differ in different parts of a cell and, in particular, may be lower in the neuropil than in the soma. This is a possible explanation for the confinement of calcium oscillations to the neuropil. [Supported in part by NSF grant BNS 85-07857, USPHS grants NS 16295 and NS 15697, and the Irma T. Hirschl Foundation.]

106. Size and Dispersity of Synaptosomes and Synaptic Vesicles from Squid (*Loligo pealei*) Optic Lobes Determined by Laser Light Scattering D. B. SATTELLE,* K. H. LANGLEY,* A. L. OBAID, and B. M. SALZBERG, *Department of Zoology, University of Cambridge, Cambridge, United Kingdom; Department of Physics, University of Massachusetts, Amherst, Massachusetts; Department of Physiology and Pharmacology, University of Pennsylvania, Philadelphia, Pennsylvania*

Laser light scattering has been used to investigate the size and dispersity of synaptosomes and synaptic vesicles isolated from squid optic lobes. Synaptosomes were prepared by the method of Pollard and Pappas (1979. *Biochem. Biophys. Res. Commun.* 88:1315) and vesicles were

purified from this fraction by sucrose-gradient centrifugation (35,000 rpm) for 2 h at 4°C using a Beckman L5-65 centrifuge. Light-scattering measurements were performed using a Coulter N4 particle size analyzer. The mean diameter (*d*) in nanometers and polydispersity ($\mu_2/\bar{\Gamma}^2$) were analyzed by the method of cumulants (Koppel. 1972. *J. Chem. Phys.* 57:4814). The particle size distribution was estimated by the method of Provencher (1982. *Comput. Phys. Commun.* 27:213). Synaptosomal fractions were highly polydisperse ($\mu_2/\bar{\Gamma}^2 = 0.5$) and mean diameters ranged from 0.5 to 2.0 μm. Analysis of the size distribution yielded two major components, a smaller particle of mean diameter 300–700 nm and a larger particle with a mean diameter in the range 2,000–5,000 nm. The heterogeneity of the synaptosomal particles detected in solution is in agreement with findings obtained by electron microscopy (Haghighat et al. 1984. *Neuroscience.* 13:527). Purified synaptic vesicle fractions also yielded complex particle size distribution data. A component with a mean diameter in the range 150–250 nm was detected, although a smaller particle (mean diameter, 40–110 nm) dominated the scattering signal. This smaller particle closely resembles in size the electron lucent vesicles seen in the majority of optic lobe nerve terminals when examined by electron microscopy (Haghighat et al. 1984. *Neuroscience.* 13:527). Osmotically induced shrinkage and swelling of the synaptosomes was detected as a change in the mean particle diameter. Depolarization by veratrine (1.0×10^{-4} M) did not result in a detectable change in the size of synaptosomal particles. Laser light scattering emerges as a rapid, nondestructive probe of the hydrodynamic properties of nerve ending particles and synaptic vesicles. [We are grateful to Langley-Ford Instruments, Inc., the Marshall and Orr Fund of the Royal Society, and the Marine Biological Laboratory. Supported by USPHS grant NS 16824.]

107. Oscillatory Activation of Calcium-dependent Potassium Channels in HeLa Cells Induced by Histamine: a Single Channel Study REMY SAUVE, CLAUDIE SIMONEAU, LUCIE PARENT, ROBERT MONETTE, and GUY ROY, *Department of Physiology and Membrane Transport Research Group, University of Montreal, Montreal, Canada* (Sponsor: Douglas C. Eaton)

The HeLa cell external membrane is known to contain calcium-activated potassium channels of small unitary conductance (Ca-K channels) and histaminergic H_1 receptors. Using the extracellular patch-clamp method, we have shown that the stimulation of the H_1 receptors by exogenous histamine induces the repetitive transient activation of the Ca-K channels. Transmembrane potential oscillations were also measured, and their occurrence could be directly correlated with the bursting activity of the Ca-K channels. In addition, single channel records were obtained that clearly showed that external calcium is not essential to the repetitive bursting channel activation evoked by histamine. Finally, we found that the permeable cyclic AMP analogue, dibutyryl cyclic AMP, was incapable of initiating single channel events such as those triggered by exogenous histamine. These results are interpreted in terms of a model in which the stimulation of H_1 receptors induces the release of calcium from internal stores via the Ins1,3,5,P_3 pathway, followed by the activation of calcium pumps. The balance between calcium release from internal stores and calcium extrusion by calcium pumps may be sufficient to generate the observed oscillations. [Supported by MRC grant MT-7769 and by an FRSQ grant.]

108. Changes in $[Ca^{++}]$ in Polarized Human Polymorphonuclear Leukocytes MARY SCANLON* and FREDRIC S. FAY, *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts*

The mechanism by which the human polymorphonuclear leukocyte (PMN) polarizes and moves in a chemotactic gradient is not fully understood. We have performed studies to determine whether changes in internal $[Ca^{++}]$ play a role in the transduction mechanism. Using FMLP, a well-known chemoattractant, we have examined the polarization of cells in suspension as well as in a linear gradient produced in the Zigmond chamber. Ca^{++} measurements were made from the ratio of fluorescences at 340 and 380 nm of cells loaded with fura-2 (F2) and studied in suspension using a Spex spectrofluorimeter or on single cells using the digital imaging microscope. PMNs were loaded with 1 μM F2 acetoxy-methyl ester for 1 h at 37°C. The

intracellular concentration of fura-2 averaged 300 μM. Addition of 10^{-9} M FMLP to cells in suspension produced an increase in [Ca^{++}] from resting levels of 70 to 200 nM within 15 s, followed by a rapid decline to 150 nM. [Ca^{++}] remained at 150 nM for several minutes. The biphasic change in [Ca^{++}] was dose dependent. Both the early transient and the sustained elevation of [Ca^{++}] were greater with increasing [FMLP]. The increase in [Ca^{++}] with increasing chemoattractant concentrations correlates with a dose-dependent increase in the percentage of cells polarized in suspension. The presence of 20 mM EGTA, added immediately before FMLP, produced the early Ca^{++} transient, but within 30 s, intracellular [Ca^{++}] returned to resting levels. This finding suggests that during FMLP stimulation, the rapid increase in intracellular Ca^{++} is from internal stores and the sustained increase in Ca^{++} over several minutes results from the influx of extracellular Ca^{++}. EGTA-treated cells did not show an increase in the percentage of cells polarized after FMLP stimulation. The sustained increase in [Ca^{++}] is apparently necessary for polarization. At the single-cell level, cells polarized in a 10^{-9} M FMLP gradient have higher intracellular [Ca^{++}] than do unstimulated cells. Within the polarized cell, there are regional differences in [Ca^{++}] from the front to the tail. We conclude that an increase in [Ca^{++}] is a necessary step for polarization. The increase in [Ca^{++}] in polarized cells is not uniform. The external chemoattractant gradient may be translated to the PMN in the form of an internal Ca^{++} gradient. [Supported by grants from the NIH (HL14523) and the Muscular Dystrophy Association.]

109. Ca^{++}-insensitive Intermediate in the Intracellular Cleavage of Fura-2

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Fura-2 (F2), the highly fluorescent Ca^{++}-binding dye, is widely used to measure [Ca^{++}] because of its many advantages over other dyes. Microinjection is obviated because F2 crosses the cell membrane as the acetoxy-methyl ester (F2-AM), is cleaved intracellularly by nonspecific esterase and is trapped within the cells. Since binding of Ca^{++} shifts the excitation spectrum of the dye to shorter wavelengths, [Ca^{++}] is calculated from the ratio of fluorescence at two wavelengths, ideally 340 and 380 nm, $R_{(340/380)}$. Thus, another advantage of F2 is the ratiometric nature of the Ca^{++} measurement, which is independent of cellular dye concentration, cell thickness, or instrument variation. There are assumptions inherent in the use of the dye, one of which is that F2-AM is completely cleaved to F2. We present evidence that the assumption of complete dye cleavage is not always valid. In many cell types, particularly human polymorphonuclear leukocytes (PMN), the cleavage of F2-AM is incomplete, resulting in a significant proportion of intracellular fluorescence that is not reflective of Ca^{++}. The presence of the Ca^{++}-insensitive fluorescence, if uncorrected, results in erroneous and unusually low [Ca^{++}]. Support for this conclusion comes from in vivo as well as in vitro studies. Upon permeabilization of F2-loaded PMN's with ionomycin or lysis of loaded cells with Triton, $R_{(340/380)}$ does not always approach the ratio predicted for Ca^{++} saturation of F2. Thin-layer chromatography of F2-loaded cell lysates indicates fluorescence at the origin associated with F2-AM, or a hydrophobic form of F2, several hours after the start of the loading procedure. In an in vitro system, the cleavage of F2-AM by commercially available esterases (Sigma Chemical Co.) results in an intermediate with relatively increased fluorescence at 380 nm. The spectral characteristics of this intermediate are related to those of tetra-methyl, mono-ethyl F2, the penultimate step in the synthesis of the dye. These results indicate that the intracellular cleavage of F2-AM is a multi-step process that produces at least one intermediate that is fluorescent but not Ca^{++} sensitive. Since the cleavage problem has arisen with all but one lot of dye tested, it is a concern of all investigators using F2. In order to accurately measure [Ca^{++}], the presence of Ca^{++}-insensitive, fluorescent intermediates in the cleavage process must be considered and measured. We present two methods that account for the uncleaved dye and accurately measure [Ca^{++}]. One requires fluorescence measurements at two wavelengths and cell lysis, whereas the other uses three wavelengths but does not require cell lysis. [Supported by grants from the NIH (HL14523) and the Muscular Dystrophy Association.]

110. Modulation of Pacemaker Currents in *Aplysia* Bursting Neurons by Intracellular Injection of Inositol 1,4,5 Trisphosphate K. P. SCHOLZ,* L. J. CLEARY,* A. ESKIN,* J. EICHBERG,* and J. H. BYRNE,* *Department of Physiology and Cell Biology, University of Texas Medical School and University of Houston, Houston, Texas* (Sponsor: S. M. Thompson)

Recent evidence indicates that receptor-mediated hydrolysis of phosphatidylinositol-bisphosphate yields two active products, diacylglycerol and inositol 1,4,5 trisphosphate (IP_3), which serve to regulate various cellular processes. In a variety of cell types, IP_3 has been shown to stimulate the release of intracellular Ca^{2+}. The mechanisms and functional roles of these agents in nerve cells, however, are poorly understood. As a first step in addressing this issue, we have examined the response of the left upper quadrant bursting (LUQB) neurons of *Aplysia* to intracellular pressure injection of IP_3. IP_3 produced a biphasic response with a total duration of 5–15 min. In freely bursting cells, the response consisted of (*a*) an initial brief depolarization (5–60 s), with a decrease in the amplitude of the interburst hyperpolarization and (*b*) a subsequent hyperpolarization accompanied by a decrease in burst frequency. In cells voltage-clamped in the pacemaker range (−35 to −45 mV), injections produced a biphasic (inward-outward) shift in the holding current (36 of 50 cells). In 14 of 50 cells, monophasic outward currents were produced. The outward component may be associated with a decreased presence of the negative-slope-resistance (NSR) characteristic described in these neurons. Control injections of D-myo-inositol yielded no consistent response ($n = 26$). The pacemaker currents underlying bursting in LUQB cells appear to be regulated by intracellular Ca^{2+}, which enters during the action potential (Kramer and Zucker. 1985. *J. Physiol.* [*Lond.*]. 362:131). Except for their time course, the currents induced by injection of IP_3 appear to be similar to the tail currents seen after a depolarizing voltage-clamp pulse from the pacemaker range. These results are consistent with the hypothesis that IP_3 leads to the release of intracellular Ca^{2+}. [Supported by NIH grant NS 19895.]

111. Calmodulin and Its Acceptor Proteins in the Basolateral Membranes of *Necturus* Enterocytes R. R. SCULLY,* J. R. DEDMAN,* W. DUBINSKY,* and S. G. SCHULTZ, *Department of Physiology and Cell Biology, University of Texas Medical School, Houston, Texas*

Calmodulin was localized in enterocytes of *Necturus maculosa* according to the method of Sternberger (1978. *Immunocytochemistry.* 104–169). Microvilli and lateral borders of these cells were highly reactive; the cytoplasm was moderately reactive. Connective tissue, endothelia, and muscularis of the submucosa were also reactive. Electron microscopy showed reaction product deposited heavily over the basolateral borders and microvilli. The mitochondrial matrix, nuclear envelope, and terminal web, and mucous granules of goblet cells were unreactive. Omission of the primary antibody or substitution of inappropriate immunoglobin (sheep anti-SRC product) for the primary antibody caused no immunoreaction product to be formed. A highly purified preparation of basolateral membranes isolated from *Necturus* small intestine according to methods modified from Boumendil-Podevin and Podevin (1983. *Biochim. Biophys. Acta.* 728:39) was examined by an SDS-gel overlay technique (Carlin et al. 1981. *Ann. NY Acad. Sci.* 356:73; Glenney and Weber. 1981. *Proc. Natl. Acad. Sci. USA* 78:2810) that identifies calmodulin acceptor proteins (CAPS) according to their abilities to bind radiolabeled calmodulin in a calcium-dependent manner. These techniques revealed the presence of two major CAPS with apparent molecular weights of 160 and 120 kD associated with that barrier. [Supported by NIH grants AM-26990, AM-07408, and GM-29323.]

112. Calcium-mediated Inhibition of Na/H Exchange in Isolated Enterocytes by Atrial Natriuretic Factor and Cyclic GMP CAROL E. SEMRAD* and EUGENE B. CHANG,* *Department of Medicine, Columbia University, New York* (Sponsor: Qais Al-Awqati)

Cyclic GMP is an important intracellular mediator of stimulus-secretion coupling in the small

intestine. However, in chicken ileum, the addition of 8-bromo-cGMP (8-Br-cGMP) has no effect on electrogenic chloride secretion. To determine whether cGMP had any direct role in electroneutral Na absorption, the effects of 8-Br-cGMP on Na uptake, intracellular pH (pH_i) and intracellular calcium (Ca_i) were investigated in isolated chicken villus enterocytes. In cells stimulated with 8-Br-cGMP (10^{-4} M) for 5 min, a significant decrease in initial ^{22}Na influx rates of 47% was observed, an effect that was not additive upon co-stimulation with amiloride (10^{-3} M). 8-Br-cGMP (10^{-4} M) also caused a persistant decrease in pH_i of ~0.1 pH unit as determined by carboxyfluorescein fluorescence. These effects were Na dependent and inhibitable with the specific calmodulin inhibitor calmidazolium (CM) (10^{-7} M). 8-Br-cGMP concomitantly stimulated an increase in cytosolic Ca_i as determined by fura-2 fluorescence. In the absence of extracellular Ca, changes in pH_i were still observed. Atrial natriuretic factor (ANF), a potent diuretic hormone, appears to stimulate cGMP in other cell systems, but little is known about its action on intestinal mucosa. Atriopeptin III (APIII, 10^{-7} M) was found to increase cGMP and not cAMP levels in isolated chicken enterocytes. Like 8-Br-cGMP, APIII (10^{-7} M) stimulated a persistent increase in Ca_i and a corresponding decrease in pH_i. Furthermore, APIII decreased intracellular pH_i in a dose-dependent fashion. These pH_i changes were absolished by pretreatment with either CM (10^{-7} M) or the isoquinoline sulfonamide compound H8 (50 μM), a selective inhibitor of cGMP-dependent kinase. However, the pH_i response to subsequent addition of the calcium ionophore ionomycin (10^{-6} M) was blocked only by CM and not by H8. We conclude that ANF and cGMP indirectly inhibit amiloride-sensitive Na/H exchange by increasing Ca_i from endogenous and exogenous sources, an event that appears to require cGMP-dependent phosphorylation. ANF may be a physiologically important regulator of gut ion transport. [Supported by NIH grants AM07330 and AM35382.]

113. Synergistic Activation of Ca^{++}-dependent Ion Channels by Ionophores and Propranolol in Ehrlich Ascites Tumor Cells: Evidence for Blockage of Cl^- Channels by Propranolol

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In Ehrlich ascites tumor cells, activation of Ca^{++}-dependent K^+ channels has been implicated in membrane hyperpolarization induced by propranolol and in volume-induced losses of cellular K^+ and Cl^-. The latter response can be mimicked by the Ca^{++} ionophore A23187. We have compared the cellular responses to propranolol and A23187 in terms of (*a*) cytoplasmic [Ca^{++}] using quin-2 fluorescence, (*b*) membrane potential, reflected by the accumulation of *N*-methyl-aminoisobutyric acid (MeAIB), and (*c*) ouabain-insensitive K^+ influx (J_{K^+}). The effects of propranolol on MeAIB uptake and J_{K^+} are consistent with membrane hyperpolarization accompanying activation of K^+ channels. Its addition gives a sharp, dose-dependent increase in MeAIB accumulation ratio (control: 36.3 ± 0.4-fold; with 133 μM propranolol: 81.0 ± 0.9-fold). J_{K^+} is similarly increased (5.5-fold), but the dose response is shifted to higher propranolol levels. Quinine (1 mM) blocks both responses. Cytoplasmic [Ca^{++}] is not altered at propranolol doses that give nearly maximal effects on MeAIB accumulation. In contrast, A23187 (10 μM) causes a transient, severalfold increase in [Ca^{++}] that peaks within 30 s and returns to control levels within 15 min. There is a concomitant transient stimulation of J_{K^+}. However, no measurable changes in MeAIB uptake occurs. This is consistent with electroneutral, parallel activation of K^+ and Cl^- channels by the ionophore (Hoffmann. 1985. *Fed. Proc.* 44:2513). The combination of A23187 with a subthreshold dose of propranolol (16 μM) induces electrogenicity. J_{K^+} is further increased and MeAIB accumulation is elevated to that found in high propranolol. Consequently, we tested the effect of propranolol (16 μM) on Cl^- influx (J_{Cl^-}). At this concentration, propranolol is without effect on MeAIB accumulation or J_{K^+}; however, initial J_{Cl^-} is inhibited by 40%. Taken together, the results are consistent with two actions of propranolol: (*a*) activation of Ca^{++}-dependent K^+ channels at normal cytoplasmic [Ca^{++}]; and (*b*) inhibition of Cl^- channel activity. [Supported by NIH grant CA28287.]

114. The Effects of Extracellular ATP and Receptor-mediated Calcium-mobilizing Agonists on Ca_i and pH_i in Rat Parotid Cells S. P. SOLTOFF, M. K. McMILLIAN,* L. C. CANTLEY, and B. R. TALAMO,* *Departments of Physiology and Neurosciences, Tufts University, Boston, Massachusetts*

We monitored Ca_i (quin-2) and pH_i (BCECF) in a suspension of rat parotid cells exposed to various Ca-mobilizing agents. ATP (10 μM to 1 mM), phenylephrine (0.1–100 μM), carbachol (carb) (0.1–10 μM), and substance P (SP) (10 fM to 1 nM) each increased Ca_i in a dose-dependent manner. The effects of carb and phenylephrine could be blocked and reversed by atropine and phentolamine, respectively. The addition of hexokinase (to deplete added ATP) reversed the effect of ATP on Ca_i. Carb and SP caused a slight (0.05 pH units) alkalinization of pH_i in cells suspended in normal media; these changes were blocked by dimethylamiloride (DMA), and appear to be mediated via the Na-H exchange system. In normal media, ATP acted in opposite fashion to these agents by slightly decreasing pH_i. In Na-free (0 Na_o) media, carb and SP produced a small decrease (0.05 units) in pH_i, but ATP caused a biphasic response: a rapid pronounced decrease in pH_i (0.3 units), followed by a slower alkalinization to a pH_i value more alkaline than the initial value in 0 Na_o. Both the acidification and the alkalinization phases were dependent on the continuous presence of ATP, were DIDS sensitive, and were not blocked by DMA. Thus, although ATP has a similar effect on Ca_i as other receptor-mediated agonists, which also appear to alter pH_i through Na-H exchange activity, ATP has effects on pH_i that are not mediated via Na-H exchange. In these cells, we also noted that amylase secretion was severalfold larger in the presence of carb than ATP. These findings suggest a novel effect of ATP, perhaps via a purinergic receptor, on rat parotid cells. [Supported in part by NIH grant F32 AM07566-02.]

115. Free Calcium Pulses During the Early Development of *Ciona* Eggs J. E. SPEKSNIJDER, D. W. CORSON,* T. H. QIU,* and L. F. JAFFE, *Marine Biological Laboratory, Woods Hole, Massachusetts*

Ascidian eggs serve as interesting systems for the study of early development, since they show a classic example of ooplasmic segregation and then develop into tadpoles within a day. We have begun to explore the role of free cytosolic calcium during the early development of *Ciona* eggs by injecting them with the calcium-specific photoprotein aequorin, and then observing their luminescence after adding sperm. Such an aequorin-loaded *Ciona* egg regularly shows an enormous pulse of luminescence that starts 1–5 min after insemination. In 30–60 s, luminescence rises to a peak level that is on the order of 10,000-fold above instrumental background. Assuming a resting free calcium level of 0.1 μM and a square-law dependence of light emission on free calcium, we estimate that free calcium rises >300-fold to a peak level of ~30 μM. The luminescence then gradually subsides, returning to the background level in ~5 min. These pulses resemble those known in medaka fish eggs as well as sea urchin eggs and presumably accompany egg activation and ooplasmic segregation. Such eggs also regularly show a remarkable series of smaller and briefer pulses in the period between the end of the activation pulse and first cleavage. These postactivation pulses occur in two series—an early group of 3–5 pulses that start soon after the activation pulse subsides (and may accompany first polar body formation), and a late group of 8–12 pulses that start ~25 min after fertilization (and may accompany second polar body formation). However, no changes in luminescence are observed during first cleavage. This first study used a simple photon counter and gave no spatial information, but we plan to re-examine these *Ciona* egg pulses with an imaging photon detector to determine just where free calcium rises in the egg. [Supported by NIH grant R01HD18818 to L.F.J.]

116. Cytosolic Free Calcium Levels During Ischemia Measured by Fluorine NMR in Perfused Rat Heart CHARLES STEENBERGEN,* ELIZABETH MURPHY, and ROBERT E. LONDON, *Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, North Carolina, and Pathology Department, Duke University Medical Center, Durham, North Carolina*

Langendorf perfused rat hearts were loaded with 5FBAPTA by perfusion with 500 ml of a solution of Krebs-Henseliet buffer containing 5 μM of the acetoxymethyl ester of 5FBAPTA. After loading, the heart was weakly beating and the mean level of cytosolic free calcium (Ca_i), reflecting a time average of systolic and diastolic Ca_i, was 630 ± 97 nM (n = 5; mean ± SE). When the heart was arrested with either 15 mM $MgCl_2$ or 25 mM KCl, the level of Ca_i fell to an average value of 252 ± 25 nM (n = 6). We were interested in determining the level of Ca_i during ischemia. After a 30-min control period, the heart was made totally ischemic for 15 min by clamping off the aortic cannula. This was followed by 30 min of reflow. ^{19}F spectra accumulated between 10 and 15 min of ischemia show an approximately fivefold increase in Ca_i to a level of 3.5–4.0 μM. This increase in Ca_i is largely reversed upon reflow, which is consistent with reversible ischemic damage. In addition, there is no loss of 5FBAPTA from the heart during reflow, ruling out any artifactual increase in Ca_i during ischemia due to leakage of 5FBAPTA into the extracellular space. Comparing ^{31}P spectra accumulated during the reflow with those of the control period, we find an increase in inorganic phosphate levels, a decrease in nucleotide phosphate levels, and a recovery to ~100% in the phosphocreatine level, which is consistent with reversibly injured ischemic tissue. In summary, these data demonstrate (*a*) that there is sufficient endogenous calcium to elevate Ca_i during ischemia to a level that could activate degradative enzymes, (*b*) that elevated Ca_i does not immediately produce lethal injury, and (*c*) that reperfusion and reoxygenation allow prompt recovery of Ca_i to control levels, even though reperfusion provides an unlimited pool of calcium that could be accumulated by the myocyte if membrane permeability to calcium were increased. [Supported by NIH grant K08-HL-01337 to C.S.]

117. Stretch Inhibition of Stimulation of ^{45}Ca Release from Skinned Muscle Fibers by Depolarizing Ion Gradients

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Depolarizing ion replacements at constant $[K^+][Cl^-]$ stimulate mechanically skinned frog twitch fibers to release ^{45}Ca, with a small Ca^{2+}-insensitive and much larger Ca^{2+}-dependent component (1985. *J. Gen. Physiol.* 86:813). If the primary locus of depolarization is sealed polarized transverse (T) tubules rather than sarcoplasmic reticulum (SR), disruption of T-SR coupling should inhibit the ionic stimulation. Previous studies suggested such disruption in highly stretched fibers; the transient stimulation of ^{45}Ca release at low $[Mg^{2+}]$ by Cl/propionate replacement at constant $[K^+]$ was inhibited selectively relative to SR stimuli (1978. *Sixth Int. Biophys. Congr. Abstr.* p. 162). The present studies tested the hypothesis more rigorously at constant $[K^+][Cl^-]$ and higher Mg^{2+} (5 mM Mg, 5 mM ATP). Fiber segments, loaded with ^{45}Ca and rinsed at sarcomere length (SL) 2.3 μm, were stimulated by choline Cl/K-methanesulfonate replacement at either 2.3 μm SL or after slow stretch to twice the length, at which SL was 2.2 μm; EGTA was added a few seconds after stimulation. ^{45}Ca release during ~1 min after stimulation decreased from 33.0% initial ^{45}Ca in unstretched segments to 9.5% in (paired) stretched segments, a reduction of 65% of total loss or ~78% of stimulated release above control loss under similar conditions. Inhibition was partially reversed in two segments with low ^{45}Ca loss during the preceding stretch, stimulation, and return to 2.3 μM SL. The results support the hypothesis that T-SR coupling is required for stimulation of Ca^{2+}-dependent ^{45}Ca release by ionic depolarization. In preliminary experiments, stretch did not similarly inhibit stimulation in 5 mM EGTA, which is very small under present conditions; the Ca^{2+}-insensitive component may include ^{45}Ca released proximal to SR stimulation. [Supported by NIH grant AM 30420.]

118. Ouabain Sensitivity of the Na-K Pump in Cultured Chick Heart Cells: Voltage Clamp and Equilibrium Binding Studies

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We used voltage-clamp and [^3H]ouabain binding techniques to study the ouabain sensitivity of the Na-K pump. Small (80–120 μm diam) aggregates of embryonic heart cells were bathed

with a modified Hanks' solution using a rapid perfusion system that allowed for solution exchange within 2 s, and voltage-clamped at −70 mV with a single-microelectrode switching clamp (Dagan model 8100). Removal of K_o caused an inward shift of the holding current, which reached a steady level in 15 s. Exposure to 1 mM ouabain for 30 s produced a similar but smaller effect. The magnitudes of the current changes with either 0 K_o or 1 mM ouabain were the same when 1 mM Ba was added to reduce K currents. Ba did not alter the ouabain-sensitive current but reduced the K_o-sensitive current. The ouabain-sensitive current was nearly constant for potentials between −70 and 0 mV (0.5 $\mu A \cdot cm^{-2}$), but decreased negative to −70 mV. This decrease could be due to (*a*) voltage dependence of the Na-K pump, or (*b*) an error in the measurement of ouabain-sensitive current when the background currents are large. The voltage-clamp technique was also used to determine the ouabain sensitivity of the Na-K pump by exposing preparations for 30–60 s to ouabain (10^{-7}–10^{-3} M). The resultant K_D (~20 μM) was higher than that obtained by equilibrium binding (30 min) of [^{3}H]ouabain (10^{-7}–10^{-5} M) to monolayers of chick heart cells (K_D = 3–5 μM). Exposure of monolayers to monensin (3 μM) caused a two- to threefold increase in Na_i and a 25% increase in equilibrium binding of 1 μM [^{3}H]ouabain, which suggests that Na_i affects ouabain binding affinity. Therefore, the higher affinity determined in equilibrium binding studies may be due to the elevation of Na_i that occurs during prolonged exposure to ouabain. [Supported by NIH grants HL27105, HL17670, HL07063, and HL07101.]

119. Effect of Basolateral pH on Membrane Voltage in *Necturus* Gallbladder Epithelium

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Studies were done to assess the effects of changes in basolateral solution CO_2/HCO_3^- concentrations on intracellular pH (pH_i) and basolateral membrane voltage (V_{cs}) in *Necturus* gallbladder epithelium. Tissues were mounted basolateral side up and the connective tissue was largely dissected away. This allowed for basolateral impalements with both conventional and pH-sensitive microelectrodes and for rapid (seconds) changes in ionic composition at the cell surface. Tissues were bathed symmetrically with a control Ringer's solution (10 mM HCO_3^-, 1% CO_2, pH 7.67); all solution changes were made on the basolateral side. Control V_{cs} and pH_i averaged −76.6 ± 1.4 mV (14) and 7.37 ± 0.03 (14), respectively. Elevation of basolateral solution CO_2 from 1 to 5% (constant HCO_3^-) caused a reversible depolarization of V_{cs} of 16.3 ± 1.7 mV (10), and a parallel decrease of pH_i of 0.32 ± 0.02 (10). Increasing CO_2 to 5% (at constant pH_o) also caused cell acidification (0.27 ± 0.02 [11]), but V_{cs} remained unchanged. Conversely, reducing [HCO_3^-] from 10 to 1 mM (constant CO_2) caused a small but significant decrease in pH_i of 0.03 ± 0.01 (14) and a depolarization of V_{cs} by 21.0 ± 0.9 mV (14). It is argued that the observed depolarizations of V_{cs} are attributable to basolateral membrane events and are primarily a result of changes in basolateral solution pH and not pH_i. Additional ion substitution studies were done where either K^+ was increased (2.5 to 25 mM) or Cl^- was decreased (98.1 to 8.1 mM) under control conditions and after a 3-min pre-exposure to 1 mM HCO_3^-. In 1 mM HCO_3^-, the K^+-induced depolarization was reduced from 41.3 ± 1.5 to 17.9 ± 2.0 mV (6), whereas the Cl^--induced depolarization was increased from 1.6 ± 0.6 to 10.0 ± 1.4 mV (6), compared with control. It is concluded that basolateral solution acidification increases the relative g_{Cl} of the basolateral membrane. [Supported by NIH grant AM19580 and by funds from the National Kidney Foundation.]

120. Intracellular Free Calcium Concentrations and Gradients in Severed and Intact Giant Spinal Axons

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We have measured the intracellular free calcium $[Ca^{2+}]_i$ in intact and cut axons of the giant Mauthner cells of the larval lamprey (*P. marinus*). The brain and spinal cord were removed from the animal and placed in organ culture and axons were iontophoretically injected with the fluorescent calcium indicator fura-2. The emitted light from a 50-μm-diam spot was measured

by a photomultiplier tube attached to a fluorescence microscope. The $[Ca^{2+}]_i$ was determined using the published method (Grynkiewicz et al. 1985. *J. Biol. Chem.* 260:3440). The resting $[Ca^{2+}]_i$ in intact axons was ~100 nM. In freshly severed axons, a gradient of calcium ranging from 2,500 nM near the cut surface to 400 nM 2.5 mm from the cut was measured. We were unable to measure closer than 0.4 mm to the cut because of the leakage of fura-2. This gradient was greatly reduced 24 h later. In these resealed axons, the $[Ca^{2+}]_i$ at 2.5 mm returned to nearly normal levels, while near the cut end it was fourfold higher. We are also measuring the $[Ca^{2+}]_i$ during longer-term recovery and during regeneration. [Supported by NIH grants HD20664 and NS18811.]

121. Purified Calcium Channels from Transverse-Tubule Membranes Incorporated into Planar Lipid Bilayers

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The dihydropyridine receptor from rabbit skeletal muscle transverse-tubule membranes was purified according to Curtis and Catterall (1984. *Biochemistry.* 23:2113), to a specific activity of 2,500 pmol [^{3}H]*PN200-110 bound per milligram protein. On sodium dodecyl sulfate-polyacrylamide gels, the purified receptor consists of three polypeptides with molecular weights of 140,000, 51,000, and 33,000, corresponding to the* α (135,000 mol wt), β (50,000 mol wt), and γ (32,000 mol wt) subunits identified by Curtis and Catterall. The purified dihydropyridine receptor was reconstituted into phospholipid vesicles (2 mg/ml phosphatidylcholine, 1.25 mg/ml phosphatidylethanolamine), and the vesicles were incorporated into planar lipid bilayers (33 mg/ml phosphatidylethanolamine, 26 mg/ml phosphatidylserine). Two types of divalent cation-selective channel, with slope conductances of 8 pS and 15–20 pS in 60 mM Ba^{++}, were observed. The 20-pS channel is blocked by Cd^{++} and by nifedipine, and appears to be similar to the 20-pS dihydropyridine-sensitive calcium channel from intact rat transverse-tubule membranes (Affolter and Coronado. 1985. *Biophys. J.* 48:341). In the presence of 6 μM Bay K8644, 2 μM nifedipine reduces the possibility of the 20-pS channel being open by >50%. The fact that two types of calcium channel are present in purified dihydropyridine receptor preparations suggests that the two calcium channel types may be closely related structurally and that the two channel types cannot be separated on the basis of dihyropyridine binding alone. [Supported by an MDA grant to J.A.T., an AHA grant-in-aid (84–879), an AHA Established Investigator Award to M.T.N., and an AHA Fellowship (Florida Affiliate) to J.F.W.]

122. Vasopressin-induced Increases of Cytosolic Calcium in Cultured Renal Tubular Cells (LLC-PK$_1$)

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Although vasopressin (V)-induced rises of cytosolic calcium (Ca_f) have been well documented in hepatocytes and smooth muscle cells, a role for increases of Ca_f in the actions of V on kidney tubules has been uncertain. *LLC-PK$_1$ cells, a permanent cell line of renal tubule origin, have* V receptors and a strong cAMP response to V. Cells were grown to confluence on glass coverslips and loaded for 30–45 min with fura-2. Exposure to fura-2 did not affect cell viability (>99%) or K^+ or ATP levels. Ca_f was estimated spectrofluorometrically on washed coverslips. Basal levels (± SEM) averaged 73 ± 3 nM (N = 32). Peak Ca_f levels induced (means for N = 6–18) were: 10^{-8} M V: 128 ± 24 nM; 10^{-7} M V: 308 ± 70 nM; 10^{-6} M V; 460 ± 62 nM. The peak Ca_f after 10^{-6} M V was reached in 42 ± 5 s followed by a return toward basal levels. The addition of a second dose of 10^{-6} M V after an initial dose of 10^{-6} M V did not raise Ca_f. Chelation of medium Ca^{++} with EGTA just before 10^{-6} M V did not reduce the response of Ca_f (peak of 359 ± 53 nM, N = 6). In contrast to V, 10^{-6} M calcitonin and PTH did not significantly increase Ca_f. The response to 10^{-6} M V was not significantly modified by prior PGE_2 (3 μM) or dibutyryl cAMP (100 μM). These data provide evidence for V-induced increases

of Ca_f via release from intracellular stores in a renal epithelial cell. [Supported by American Heart Association grant-in-aid 83-1200 and NIH RCDA AM-01337.]

123. Voltage-sensitive Influx of Calcium into Central and Peripheral Neurons STANLEY A. THAYER,* SHAWN N. MURPHY,* TERESA M. PERNEY,* LANE D. HIRNING,* KATHERINE M. HARRIS,* and RICHARD J. MILLER,* *Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois* (Sponsor: M. Villereal)

Cytosolic calcium, $[Ca^{2+}]_i$, was measured in single neurons grown in primary culture using the fura-2 ratio technique. Peripheral neurons were cultured from the rat dorsal root (DRG) and superior cervical ganglia (SCG), and cultures from the CNS included neurons from the rat hippocampus (H) and the mouse striatum (S), cerebellum, and cortex. All of the neurons studied responded to depolarization, produced by perfusion with media containing 50 mM K^+, with a rapid rise in $[Ca^{2+}]_i$ from resting levels below 100 nM to levels typically >400 nM. Substitution of choline for Na^+ in the media did not significantly alter the peak rise in $[Ca^{2+}]_i$ in either H or SCG neurons. However, the rate of return of $[Ca^{2+}]_i$ to basal levels was increased considerably in the SCG neurons, while the time course of the H neurons was not noticeably affected. Neurons were tested for their sensitivity to dihydropyridine (DHP) drugs. Cells were perfused with 10 mM K^+, a near-threshold depolarization, which produced a small transient rise in $[Ca^{2+}]_i$; subsequent addition of 1 μM BAY K8644 produced a two- to sixfold increase in $[Ca^{2+}]_i$ in all six neuronal types. In addition, depolarization-induced increases in $[Ca^{2+}]_i$ in neurons from the H and SCG were found also to be inhibited by 1 μM nitrendipine, a DHP antagonist. These data are consistent with the idea that the DHP binding sites found in brain represent functional calcium channels. In S neurons, $[Ca^{2+}]_i$ could be raised significantly above basal levels by the addition of the excitatory amino acid *N*-methyl-D-aspartic acid (NMDA). This effect was dose dependent, with a threshold [NMDA] of <1 μM. The NMDA response could be competitively blocked by the NMDA receptor blocker AP5 in a stereospecific manner and could be noncompetitively blocked by phencyclidine and Mg^{2+}. [Supported by USPHS grants MH40165, DA02121, and DA02575, and by grants from Miles Co. and the Brain Research Foundation. R.J.M. is a Guggenheim Fellow.]

124. Growth Factors Stimulate Transient Increases in Free Calcium (Ca_i) in Human Fibroblasts: Digital Image Analysis with Fura-2 and a Microcomputer ROBERT W. TUCKER and HARRY LOATS,* *Johns Hopkins Oncology Center, Baltimore, Maryland, and Loats Associates, Inc., Westminster, Maryland*

To investigate whether intracellular calcium (Ca_i) can stimulate mitogenic events in fibroblasts, we used a fluorescent calcium probe (fura-2) and digital image analysis to analyze the spatial-temporal changes of Ca_i in PDGF-stimulated fibroblasts. Human FeSin cells were loaded with 1 μM of fura-2 acetoxy methyl ester and analyzed using quartz optics and 340 nm/380 nm excitation, 500 nm emission. Intracellular fura-2 distribution (380 nm excitation) indicated uniform loading of the cytoplasm and nucleus with occasional stained cytoplasmic organelles and unstained vesicular areas. Using a ratio of cell images excited at 340 nm and 380 nm, we found that unstimulated cells had an average basal Ca_i of ~25 nM with generally higher free calcium concentrations in the perinuclear area. When stimulated with PDGF (4 U/ml), Ca_i increased first in the peripheral cytoplasm and later spread to the nucleus and the perinuclear area. The transient Ca_i increase was as high as 120 nM during the first 2 min after PDGF stimulation. These results suggest that the mechanism responsible for an increase in free calcium after stimulation with PDGF cannot be a generalized production of an activator (e.g., IP_3) that synchronously releases calcium from the entire endoplasmic reticulum in FeSin cells. Further studies are necessary to define the relationship between this spatial-temporal increase in Ca_i and subsequent mitogenesis.

125. Na/H Exchange in Cultured Rat Aortic Smooth Muscle Cells Is Stimulated by Angiotensin II G. VALLEGA,* M. CANESSA, B. C. BERK,* T. A. BROCK,* M. A. GIMBRONE, JR.,* and R. W. ALEXANDER,* *Departments of Medicine and Pathology, Brigham and Women's Hospital, Boston, Massachusetts*

Angiotensin II (ang II) is a potent vasoconstrictor that stimulates Na^+ influx in vascular smooth muscle cells (VSMC). To investigate the role of Na/H exchange in mediating ang II–stimulated Na^+ influx, we studied amiloride- (Na/H exchange inhibitor) sensitive ^{22}Na influx in cultured rat aortic VSMC (passages 10–20). Initial rates (2 min) of ^{22}Na influx were measured in buffer (pH 7.0) containing 1 mM ouabain and 0.1 mM bumetanide to inhibit the Na-K pump and Na-K-Cl cotransport, respectively. Preincubation of VSMC for 30 min in Na^+-free buffer (130 mM choline, pH 7.0) decreased cytosolic pH from 7.1 (basal) to 6.75, as measured with the pH-sensitive fluorescent dye BCECF. Under these conditions, the basal rate of ^{22}Na influx (20–50 nmol/mg prot·min) was dependent on external Na (Na_o = 10–130 mM) with K_m = 40–50 mM and V_{max} = 30–40 nmol/mg prot·min. Amiloride markedly attenuated ^{22}Na influx at all Na_o and a Dixon plot of the amiloride effect revealed it to be a competitive inhibitor (K_i = 10–20 μM). Ang II- (20 nM) stimulated ^{22}Na influx occurred within 2 min, was maximal (1.6-fold increase) at 20 min, and was completely inhibited by the ang II receptor antagonist, sar^1,ile^8-ang II. Ang II–stimulated ^{22}Na influx was almost completely inhibited (90%) by amiloride (0.3 mM). These observations suggest that Na/H exchange is a major pathway for Na^+ influx in cultured VSMC and is stimulated by vasoconstrictors such as ang II.

126. Calcium Channels from Cardiac Sarcoplasmic Reticulum Incorporated into Bilayer Membranes PETER M. VASSILEV* and JOSEPH R. HUME, *Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan*

A Ca^{++} channel mechanism may be responsible for the release of Ca^{++} from cardiac sarcoplasmic reticulum (SR) similar to that demonstrated for skeletal muscle SR (Smith et al. 1985. *Nature [Lond.]*. 316:446). We have examined the properties of Ca^{2+} channels from calf heart SR reconstituted in bilayers formed at the tip of patch electrode pipettes using the dipping technique (Suarez-Isla et al. 1983. *Biochemistry*. 22:231). Bilayers were formed from lipids and proteolipids derived from cardiac SR membranes in the presence of various Ca^{++} gradients (3.6–50 mM). Several properties of SR Ca^{++} channels correlate with the Ca^{++}-releasing properties of SR membranes analyzed in other preparations: (*a*) activation by micromolar Ca^{++} at the side corresponding to the cytoplasmic surface, (*b*) significant attenuation of activity by ruthenium red (0.5 μM) and ryanodine (12 μM), and (*c*) insensitivity to sarcolemmal Ca^{++} blockers like nifedipine (40 μM). In most of our experiments, two distinct sizes of current amplitude were observed (~8–10 pS and 20–25 pS, with symmetrical solutions containing 20 mM Ca^{++}). Occasionally, larger amplitude events exhibiting bursting activity were also observed. All three conductances exhibit properties *a–c* and therefore appear to be involved in mediating Ca^{++} release from cardiac SR. The different conductances observed may represent different populations of SR Ca^{++} channels or different conductance states of a single channel population. [Supported by NIH grant HL30143 and by the AHA.]

127. $[Ca^{2+}]_i$ Transients Measured with Fura-2 in Single Isolated Rat Ventricular Cells W. G. WIER, S. W. ROBINSON, and E. MARBAN, *Department of Physiology, University of Maryland at Baltimore, Baltimore, Maryland*

Single rat ventricular cells were loaded with fura-2 by exposure to fura-2 acetoxy methyl ester and studied using an inverted microscope equipped with epifluorescence illumination, an intensified silicon intensifier target camera (ISIT), and a photomultiplier tube (PMT). Fluorescence emission at 510 nm could be recorded simultaneously with the ISIT and with the PMT, which was masked such that it received light from only a small region of the cell (5 μm diam). The cells were illuminated alternately during successive physiological contractions with light at three wavelengths: 340, 360, and 378 nm. Digital analysis of video images obtained at 33-ms

intervals during these contractions revealed that the $[Ca^{2+}]_i$ transient is uniform spatially, within the resolution of the fluorescence microscope. The PMT revealed the time course and amplitude accurately. Analysis of video data revealed the rate and extent of shortening. Compared with $[Ca^{2+}]_i$ transients calculated from aequorin luminescence in multicellular cardiac preparations, $[Ca^{2+}]_i$ transients calculated from fura-2 fluorescence in single cells rise more rapidly, but fall more slowly. The peak $[Ca^{2+}]_i$ attained during physiological contractions (400 nM) is at the low end of the range obtained with aequorin (Wier and Yue. 1986. *J. Physiol.* [*Lond.*]. In press). The extent to which these differences are related to the different Ca indicators, rather than to the different experimental conditions, is being investigated. [Supported by NIH grant HL29473. W.G.W. is an Established Investigator of the American Heart Association.]

128. Ca^{2+} and Fura-2 in the Nuclear Environment of Isolated Smooth Muscle Cells

DAVID A. WILLIAMS* and FREDRIC S. FAY, *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts*

It appears that nuclear $[Ca^{2+}]$ may be regulated at a level that differs from that of the rest of the cell (Williams et al. 1985. *Nature* [*Lond.*]. 318:558). This independence may be seen as a strategy to prevent fluctuations in the activity of nuclear-mediated function during the elevation of cytosolic $[Ca^{2+}]$, particularly during smooth muscle contraction. We have undertaken a series of experiments using fura-2–loaded smooth muscle cells and fura-2 (pentapotassium salt) in Ca-buffered solutions to investigate the potential interaction of fura-2 and/or Ca^{2+} with nuclear constituents. The presence of large concentrations of Na_2ATP, MgATP (20–30 mM) histones, and single- or double-stranded DNA (Sigma Chemical Co.) had no effect on the spectral characteristics of fura-2 in vitro (as viewed with a Spex fluorolog 211 fluorimeter). Calmodulin, reported to be present in significant concentrations within the nucleus, also produced no spectral change in cuvette concentrations between 5 and 120 μM. No change in dye properties was noted over a broad range of pH (6.5–8.0). Localized increases in cell permeability induced by supermaximal stimuli allowed for the full and rapid release of intracellular fura-2, which indicates that the dye does not bind to internal membranous systems. Lightly homogenized cell nuclei (sperm nuclei, Sigma Chemical Co.) totally quenched fura-2 fluorescence in the presence or absence of Ca^{2+}. This association may explain the spatial heterogeneity in Ca^{2+} levels monitored within the nucleus of a single smooth muscle cell viewed using the digital imaging microscope. Many divalent (Ba, Cd, Sr, Mg, Zn) but not monovalent (K, Na, Li) cations, as expected, were able to induce specific changes in fura-2 fluorescence after binding to the dye, although with slight differences in isosbestic point and dissociation constant. In order to investigate potential heavy metal influences in smooth muscle, the heavy metal chelator TPEN was added to fura-2–loaded cells: ratio images (340/380 nm excitation) showed that the nuclear-cytoplasmic $[Ca^{2+}]$ difference did not diminish. Ionomycin, at levels leading to equilibration of intracellular and extracellular $[Ca^{2+}]$ (2–5 μM, with the precise concentration dependent on cell density), was able to dissipate the nuclear-cytoplasmic fluorescence difference in single cells in the presence of low (200 nM) or normal (1.8 mM) extracellular Ca^{2+}, which suggests that a true Ca^{2+} gradient does exist. An increasing number of observations made with a variety of cell types (including 3T3 fibroblasts, neutrophils, bladder epithelial cells) indicate that the nuclear-cytosolic difference in $[Ca^{2+}]$ may be a general cell property. We have not been able to artifactually reproduce this difference in vitro. [Supported by grants from the NIH (HL14523) and the MDA, and by fellowships from the Australian National Heart Foundation and American Heart Association (Massachusetts Affiliate) to D.A.W.]

129. Maximum Cell Shortening in Single Smooth Cells Requires Only Small Transient Changes in Cytosolic Ca^{2+}

DAVID A. WILLIAMS, KEVIN E. FOGARTY, and FREDRIC S. FAY, *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts*

A combination of the technology of digital imaging microscopy and the use of new Ca-sensitive fluorescent probes such as fura-2 has allowed us to make determinations of intracellular

$[Ca^{2+}]$ in freshly isolated smooth muscle cells. The collection of a series of fluorescent video images at alternating wavelengths (340 and 380 nm excitation), during the depolarization-induced contraction of a single cell, has provided information about the changes in cytosolic $[Ca^{2+}]$ and cell length that underlie this process. Ca^{2+} rose from resting levels (140 nM) several seconds before the onset of cell shortening, peaking between 450 and 850 nM (mean 725 ± 62 nM, $n = 12$). Ca^{2+} had returned to resting levels before the cells had re-elongated by >20–30% of their peak shortening (cells shortened to <30–40% of initial length). The duration of the Ca^{2+} transient showed a direct dependence on the time of application of the depolarizing stimulus (K^+ depolarization), whereas the magnitude did not. Local, supermaximal electrical stimulation resulted in localized contractions, followed by waves of Ca^{2+} traversing the remainder of the cell. The nuclear environment seemed to be shielded from the changes in cytosolic Ca^{2+}, as nuclear Ca^{2+} underwent little or no change during smooth muscle cell contraction. This observation provides direct physiological support for the recent contention that the cell nucleus is able to regulate its Ca^{2+} concentration at a level different from that found in the cytoplasm. The results obtained with fura-2 are consistent with the involvement of powerful regulatory mechanisms (such as a sarcolemmal Ca-ATPase and Ca-dependent Ca channel inactivation) for controlling intracellular $[Ca^{2+}]$ in smooth muscle and also suggest that myosin light-chain kinase necessarily has a very strong affinity for the Ca-calmodulin complex formed in the contractile cascade. [Supported by grants from the NIH (HL14523) and the MDA and by fellowships from the Australian National Heart Foundation and American Heart Association (Massachusetts Affiliate) to D.A.W.]

130. Calcium Antagonists Protect Renal Tubular Cells from Anoxic Damage In Vitro PATRICIA D. WILSON, M. BURNIER,* A. SCHIEPPATI,* and U. SCHWERTSCHLAG,* *Department of Physiology/Biophysics, UMCNJ-Rutgers Medical School, Newark, New Jersey; Renal Division, University of Colorado Health Sciences Center, Denver, Colorado; Renal Division, Duke University Medical Center, Durham, North Carolina*

Calcium has been implicated as a causative factor in the impairment of renal function induced by ischemia. To evaluate whether calcium has any direct action on renal tubular elements in the absence of vascular effects, the effects of anoxia were studied in cultured and freshly isolated renal tubular cells in the presence and absence of the calcium channel blockers verapamil (V) and nifedipine (N) and the calmodulin inhibitors trifluoperazine (TFP) and W7. Primary cultures of individually microdissected rabbit proximal convoluted tubules (PCT), S_1 and S_2 segments, proximal straight tubules (PST), thick ascending limbs of Henle's loop (TAL), cortical collecting tubules (CCT), and highly enriched freshly isolated proximal tubules obtained by Percoll centrifugation were subjected to 5–45 min anoxia in 95% N_2/5% CO_2, and cell viability was examined immediately after the insult and after varying periods of reoxygenation. Cell death was determined by vital dye (nigrosine, 50 mg/ml) uptake index lactate dehydrogenase release, and ultrastructural analysis. Functional integrity was substantiated by the normal adenylate cyclase response to segment-specific hormones. After 45 min anoxia and 5 h incubation in normal, reoxygenated, calcium-containing media, all cultured PCT, PST, TAL, and CCT cells were dead. Cell survival after 45 min anoxia and 5 h "reflow" was significantly improved by removal of calcium for the initial 2 h of reincubation (PCT, 37 ± 7%; PST, 60 ± 6%; TAL, 31 ± 9%; CCT, 38 ± 4%) or by the addition of 5×10^{-7} M V (PST, 60 ± 8%; CCT, 57 ± 6%) and 10^{-6} M N (PST, 33 ± 7%; CCT, 41 ± 11%). A significant improvement in ultrastructural integrity was also seen in freshly isolated PST subjected to 15 min anoxia in the presence of V or N. TFP (5×10^{-7} M) and W7 (5×10^{-7} M) also afforded protection from post-anoxic cell death in cultured PST cells (58 ± 3 and 62 ± 3%, respectively). It is concluded that prevention of calcium entry and inhibition of calcium-calmodulin interactions are potent means of attenuating anoxia-induced cell death in renal tubular cells. [Supported in part by contract DAMD 17-83-C-3146 from the Department of the Army.]

131. Identification of Single Calcium Channels in Cells Isolated from Mesenteric

and Cerebral Arteries JENNINGS F. WORLEY III* and MARK T. NELSON, *Department of Pharmacology, University of Vermont College of Medicine, Burlington, Vermont* (Sponsor: K. Magleby)

Single smooth muscle cells were enzymatically isolated from both the mesenteric artery and the basilar artery of the rabbit. After isolation, single smooth muscle cells from both types of arteries were relaxed in physiological solutions (cell length ranged between 100 and 200 μm, diameter ~5 μm around the nucleus, with the ends tapering to <1 μm). Single cells from the mesenteric artery contracted in response to 10 μM norepinephrine (NE), whereas the same concentration of NE did not affect single cells from the basilar artery. Serotonin and histamine (10 μM) induced the basilar artery cells to contract. High potassium induced contraction of both cell types. Currents through single calcium channels were recorded in excised membrane patches and in planar lipid bilayers. With barium as the charge carrier (80 mM), two sizes of single channel currents were observed in the mesenteric artery cells and the basilar artery cells, corresponding to single channel conductances of 8 and 15, and 12 and 20 pS, respectively. These channels were highly selective for barium over sodium, potassium, chloride, *N*-methyl-D-glucamine, and cesium. The activity of these calcium channels increased as the membrane potential was depolarized beyond −40 mV. In conclusion, we have developed a procedure for isolating physiologically responsive single cells from both the mesenteric artery and the cerebral artery. Furthermore, we have identified single calcium channels in both types of vascular smooth muscle cells. [Supported by a grant-in-aid 84-879 from the American Heart Association (AHA). M.T.N. is an Established Investigator of the AHA. J.F.W. is a Fellow of the AHA, Florida Affiliate.]

132. Activation of an Insect Hindgut Adenylate Cyclase by the Neuropeptide Proctolin M. S. WRIGHT,* B. J. COOK, and G. M. HOLMAN,* *United States Department of Agriculture, Agriculture Research Service, College Station, Texas*

Adenylate cyclase (AC) derived from a 10,000 *g* supernatant of homogenized hindgut tissue from *Leucophaea maderae* (LM), is stimulated by low Ca^{2+} concentrations (<100 μM) and forskolin. The enzyme is inhibited by elevated Ca^{2+} concentrations (>100 μM). In the presence of 9.6 mM Mg^{2+}, the addition of Ca^{2+} caused a biphasic response, such that AC activity increased until the Ca^{2+} concentration reached 100 μM, and then decreased ~50% as the Ca^{2+} concentration was increased to 200 μM. The addition of Mn^{2+} to the reaction mixture caused a slight initial stimulation of basal enzyme activity. However, unlike Mg^{2+}, the Mn^{2+} did not show any appreciable stimulation or inhibition of the enzyme as the Ca^{2+} concentration was increased. Although AC activity was found in all tissues assayed, the highest levels were detected in muscle tissues like the superior longitudinal muscle found in the hindgut. Thus, it is possible that a good part of the AC activity seen in the hindgut preparations comes from the muscular component. The myotropic pentapeptide proctolin exerted a direct multiphasic effect on LM hindgut AC activity. Proctolin (10 pM) caused an initial 50% stimulation in AC activity and, as the peptide concentration reached 50 pM, the enzyme activity reached a value 700% above basal activity. The myotropic activity of proctolin may regulate hindgut muscle contraction through a second-messenger system that involves cyclic AMP.

133. Role of Increased Permeability for K^+ and Cellular Ca^{2+} Mobilization in the Hyperpolarizing Responses to Intestinal Secretagogues in Cultured Epithelial Cells (Intestine 407) TOSHIHIKO YADA* and YASUNOBU OKADA,* *Department of Physiology, Faculty of Medicine, Kyoto University, Kyoto, Japan, and Department of Pharmacology, College of Veterinary Medicine, Cornell University, Ithaca, New York* (Sponsor: Geoffrey W. G. Sharp)

Cultured epithelial cells (intestine 407) derived from fetal human small intestine had a resting potential of about −20 mV and were hyperpolarized to about −80 mV in response to mechanical or electrical stimuli, and to the application of the intestinal secretagogues acetylcholine (ACh),

histamine, serotonin, and vasoactive intestinal peptide (VIP) (Yada and Okada. 1984. *J. Membr. Biol.* 77:33). The membrane desensitization to one of these secretagogues after repeated application did not prevent the responses to other secretagogues. Atropine selectively inhibited the hyperpolarizing responses to ACh, and diphenhydramine inhibited the hyperpolarizing responses to histamine. From these results, we concluded that the hyperpolarizing responses to ACh, histamine, serotonin, or VIP are mediated by specific receptors, including muscarinic for ACh, and H_1 receptors for histamine. A reduction of the external Na^+ concentration to 3 mM or external Cl^- concentration to 30 mM had little effect on the hyperpolarizing responses. By contrast, the peak potentials of the hyperpolarizing responses were markedly dependent on $[K]_o$, varying linearly with the $\log[K]_o$ with a slope of 57 mV. The reversal potential of the hyperpolarizing responses was around −90 mV, which was close to the equilibrium K^+ potential (−89 mV) obtained using K^+-selective microelectrodes. These results strongly suggest that the hyperpolarizing responses are caused mainly by an increase in the K^+ conductance. Quinine and quinidine, inhibitors for Ca^{2+}-activated K^+ channels, inhibited the hyperpolarizing responses. The Ca^{2+} ionophore A23187 induced prolonged hyperpolarization. Intracellular injection of Ca^{2+} brought about hyperpolarizing responses. An increase in the intracellular Ca^{2+} concentration was demonstrated during hyperpolarizing responses to intestinal secretagogues by means of Ca^{2+}-selective microelectrodes. It is conceivable that the receptor stimulation by intestinal secretagogues mobilizes cellular Ca^{2+}, which in turn operates the Ca^{2+}-activated K^+ channel, resulting in the hyperpolarizing membrane response.

134. Rapid Effects of Phorbol Ester on Cytoplasmic Calcium in Human Platelets

KEN-ICHI YOSHIDA,* GEORGE DUBYAK,* FREDDY STARK,* and VIVIANNE T. NACHMIAS,* *Department of Anatomy, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania*

A rise in cytoplasmic Ca^{2+} and activation of Ca^{2+}–phospholipid-dependent kinase (C-kinase) are both important in the activation of human platelets. Phorbol esters are known to activate C-kinase directly, and have been shown to potentiate platelet secretion synergistically with free Ca^{2+}. However, it has been reported that after preincubation of platelet with phorbol esters for several minutes, the thrombin-induced Ca^{2+} transient is inhibited. In this study, we used fura-2–loaded platelets and found that phorbol 12-myristate, 13-acetate (PMA), but not the inactive analogue, added 10 s before the agonist inhibits the Ca^{2+} transient following thrombin (0.025 or 0.5 U/ml) or ADP (10 μM) in a dose-dependent fashion. Preincubation with even 1 nM PMA for 10 s inhibited the Ca^{2+} signal from 0.025 U/ml thrombin by 50%, and 20 nM PMA completely inhibited the Ca^{2+} signal from ADP. When PMA was added 10–15 s after thrombin, it increased the rate at which the Ca^{2+} transient returned to the baseline and caused an apparent overshoot; a significant effect was observed at 0.5 nM and at 50 nM increased the rate of return by ~50%. Inhibition of the interaction of the Ca^{2+} transient correlated with inhibition of shape change and of myosin association with the cytoskeleton. However, PMA added after thrombin or ADP did not reverse these functional responses. Unlike PGE_1, PMA does not inhibit phosphorylation of myosin light chain as well as the 40-kD polypeptide. We are examining the effect of PMA added after the agonist in terms of the interaction of adenylate cyclase caused by thrombin vs. calcium removal via the plasma membrane or the internal membrane system. It appears that in this sequential system, C-kinase activation inhibits early responses while enhancing later ones. [Supported by grant HL 15835 to the Pennsylvania Muscle Institute and by the Thomas McCabe Foundation.]

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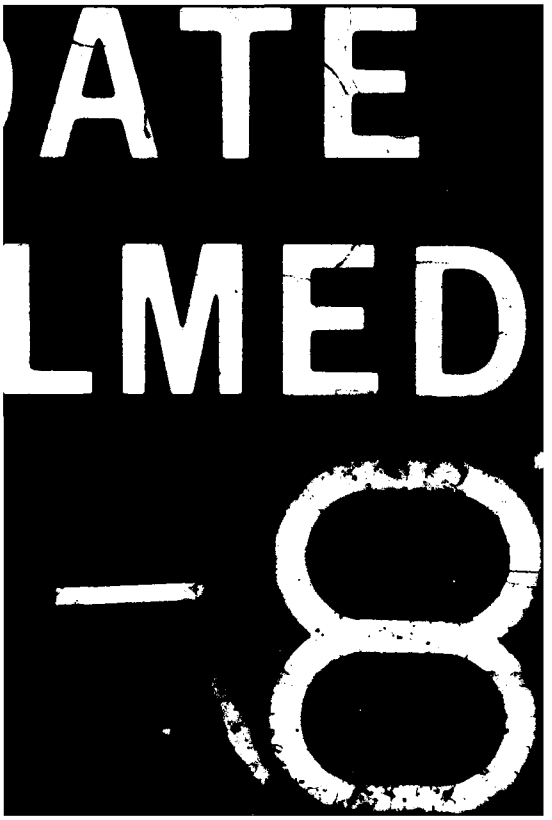
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